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1. Your reference	P71950 TAC/AB	**-1 NOV 1996**
2. Patent application number *(The Patent Office will fill in this part)*		**9622852.3**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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APPLICATION FILED 27·8·99

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

Great Britain

4. Title of the invention

Treatment of Intimal Hyperplasia

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

J A Kemp & Co
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Patents ADP number *(if you know it)*

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	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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a) *any applicant named in part 3 is not an inventor, or*
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Description	46
Claim(s)	5
Abstract	None
Drawing(s)	12

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Priority documents

Translations of priority documents

Statement of inventorship and right
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11.

I/We request the grant of a patent on the basis of this
application

Signature
J. A. KEMP & CO

Date 1 November 1996

12. Name and daytime telephone number of
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CRESSWELL, Thomas Anthony
0171 405 3292

TREATMENT OF INTIMAL HYPERPLASIA

The present invention relates to the treatment and prevention of intimal hyperplasia of blood vessels, and other similar conditions.

Intimal hyperplasia is the increase in the number of cells
5 between the endothelium and internal elastic lamina of a blood vessel, particularly an artery. Intimal hyperplasia is often caused by smooth muscle cell (SMC) proliferation in the blood vessel wall. When intimal hyperplasia occurs after surgery, e.g. arterial surgery, restenosis may result. Thus,
10 when an obstruction in the artery has been cleared, intimal hyperplasia occurring after surgery may lead to the artery becoming obstructed again. Similarly, intimal hyperplasia may lead to intimal thickening, that is to say an increase in the thickness of the intimal layer found between the internal
15 elastic lamina and the endothelium. The present invention seeks to treat and/or prevent all of these conditions, in as much as they arise from intimal hyperplasia.

Proliferation of arterial smooth muscle cells can occur when a blood vessel, e.g. an artery, is deformed or disturbed. For
20 example, intimal hyperplasia can occur following balloon angioplasty procedures which stretch arteries that have become occluded, following coronary bypass grafts in which a vein is anastomosed to an artery, and following surgical anastomosis in general.

25 Intimal thickening, smooth muscle cell (SMC) proliferation and restenosis remain major problems after coronary angioplasty, affecting more than a third of patients within six months after the operation. To date, numerous methods of treating or preventing intimal hyperplasia have been tested,
30 but none have been clinically satisfactory.

Surprisingly, the present inventors have now found that vascular endothelial growth factor (VEGF) can be used to combat intimal hyperplasia. The inventors placed a collar around the outside of the artery of a rabbit. This procedure
5   normally causes intimal hyperplasia in the rabbit artery which is similar to the restenosis that can occur in human arteries following angioplasty. When the collar was used to deliver DNA encoding VEGF to the arterial wall using a plasmid/liposome vector, the VEGF gene was overexpressed in
10   the arterial wall, even in the endothelial layer. Intimal hyperplasia was inhibited. It has been found that the adventitial collar is suitable for arterial gene transfer with all tested gene delivery systems.

VEGF is known to play a part in angiogenesis, where it
15   stimulates the division of vascular endothelial cells (EC), increases endothelial permeability and acts as an endothelial "survival factor" in retinal vessels. This property has led to its use in repairing arteries whose endothelia have been damaged during surgery. It has also been shown that VEGF, in
20   the form of recombinant protein or when expressed from a plasmid, can induce the development of new blood vessels when injected intra-arterially into ischaemic limbs.

VEGF has previously been observed to promote the development of blood vessels, and its inhibition of the proliferation of
25   arterial smooth muscle cells shows that it has properties very different to its previously known properties.

VEGF mediates its known effects via specific high-affinity tyrosine kinase receptors flk-1/KDR and flt-1 which are only expressed on EC and monocytes, and the inventors consider it
30   likely that the effects of VEGF in the inhibition of hyperplasia are also mediated through the same receptors.

An advantage of VEGF is therefore that, unlike many other growth factors and cytokines suggested for the treatment of

intimal thickening and restenosis, the effects of VEGF are
more specific to EC since, in the absence of monocytes, high
affinity VEGF receptors in the arterial wall are only
expressed on EC.   More specifically, the inventors have
5   found that the mechanism of VEGF's inhibition of intimal
hyperplasia is at least partly via the nitric oxide (NO)
pathway, as administration of the NO synthesis inhibitor L-
NAME counteracts VEGF's effects on intimal hyperplasia.   It
is also possible that VEGF has other biological effects that
10   contribute to its inhibition of intimal hyperplasia.   In
addition, the inventors have found that VEGF overexpression
is associated with increased expression in the arterial wall
of inducible nitric oxide synthase (iNOS).

Accordingly, the present invention provides:

15   Use, in the manufacture of a medicament for the
treatment or prevention of intimal hyperplasia of blood
vessel, of vascular endothelial growth factor (VEGF) protein
or a nucleic acid encoding VEGF protein.

An implant comprising VEGF protein or a nucleic acid encoding
20   VEGF protein.

A method of treating or preventing intimal hyperplasia
of a blood vessel comprising administering to a patient in
need of such treatment an effective non-toxic amount of VEGF
or of a nucleic acid encoding VEGF.

25   A kit for the treatment on prevention of intimal
hyperplasia which comprises: (i) VEGF protein or nucleic acid
as defined herein; and (ii) an implant into which the VEGF
protein or nucleic acid may be introduced.

BRIEF DESCRIPTION OF THE DRAWINGS

30   **Figure 1** (A)   Intima/media ratios in VEGF-transfected and

LacZ-transfected arteries.

(B)   Analysis of VEGF-transfected arteries and LacZ
       transfected arteries by RT-PCR

Figure 2   Micrographs of rabbit carotid arteries 7 days after
5   VEGF or LacZ transfection.

Figure 3   Effect of NO synthesis inhibitor L-NAME on intimal
                thickening in VEGF-transfected arteries and LacZ-
                transfected arteries.

Figure 4   (A)   VEGF induces phosphorylation of a major 205kD
10   protein corresponding to VEGF receptor.
                (B)   Time course of tyrosine phosphorylation.
                (C)   Time course of nitrite production after
                       addition of VEGF to HUVEC.
                (D)   Dose response of nitrite production after
15   addition of VEGF to HUVEC.

Figure 5   Neointimal thickening and proliferation indices in
                rabbit carotid arteries after positioning of a
                silastic collar around the carotid artery.

Figure 6   Micrographs depicting the effects of gene transfer
20   into collared rabbit carotid arteries.

Figure 7   Schematic view of a preferred implant (collar) of
                the invention in place around a blood vessel
                (coronal section).

Figure 8   Schematic view of a preferred implant (collar) of
25   the invention in place around a blood vessel
                (longitudinal section).

Vascular endothelial growth factor (VEGF) is a naturally
occurring protein. In humans, at least four forms exist, of

121, 165, 189 and 206 amino acids. The cDNA and amino acid
sequences of the four forms of human VEGF are given in Houck
et al (1991) Molecular Endocrinology vol 5, No. 12, pages
1806-1814. A partial genomic sequence is also given. The

5   cDNA sequence of human VEGF is also given in Leung *et al*
(1989) Science vol. 246, pages 1306-1309, together with the
bovine VEGF cDNA sequence. The DNA and encoded amino acid
sequences of the four known forms of human VEGF are also
given in the section entitled "Sequence Information".

10   These four forms are referred to herein as VEGF-121, VEGF-
165, VEGF-189 and VEGF-206. It should be understood that
this numbering refers to the number of amino acids in the
mature protein in each case. The translated protein also
includes a 26 amino acid presequence which, in nature, is

15   cleaved off during intracellular processing. Thus, the amino
acid sequences given in the "Sequence Information" section
for VEGF-121, VEGF-165, VEGF-189 and VEGF-206 actually
comprise 147, 191, 215 and 232 amino acids respectively.

Herein, references to these VEGF protein sequences are to be

20   understood to refer both to sequences comprising the
presequence and sequences lacking the presequence. VEGF
proteins with and without the presequence are suitable for
the practice of the invention.

Similarly, references to VEGF nucleic acid (DNA and RNA)

25   sequences relate to both sequences encoding the presequence
and sequences that do not encode the presequence.

It should also be noted that Houck *et al* gives the sequence
of VEGF-165 as including the amino acid asparagine (N or Asn)
at position 141 (115 in the notation of Houck *et al* which

30   begins at the beginning of the mature protein). Houck *et al*
gives this amino acid as lysine (K or Lys) in VEGF-121, VEGF-
189 and VEGF-206, and the cDNA sequence (of VEGF-206) quoted
in Houck *et al* supports this. Therefore, in the amino acid

sequences of the invention, the amino acid at position 141 may be asparagine (N or Asn) or lysine (Lys or K). Each amino acid is encoded by the appropriate triplet codon in nucleic acid sequences of the invention (for DNA, these
5   codons may be AAA or AAG for lysine and AAT or AAC for asparagine). This applies especially to VEGF-165.

The four forms are encoded by the same gene but generated by alternative splicing at the RNA level. Thus, there is a full length form of human VEGF and three known truncated forms.
10   VEGF-121 and VEGF-165 are soluble and are secreted forms. Similarly, the 26 amino acid presequence is hydrophobic and is believed to decrease the solubility of the protein. Thus, forms of VEGF without the presequence are preferred, as they are expected to have higher solubility. All forms of VEGF
15   are suitable for the practice of the invention, though secreted forms are preferred. VEGF proteins suitable for the practice of the invention may also originate from other species, although human VEGF is preferred. For example, mouse, rabbit and cow VEGF have been cloned and their
20   sequences are available.

For reference, it should be noted that VEGF-121, 165, 189 and 206 are also referred to in the art as VEGF-120, 164, 188 and 205. VEGF proteins and nucleic acids (DNA and RNA) are suitable for the practice of the invention.

25   In the practice of the invention, it is preferred to use VEGF DNA having the sequence of SEQ ID No. 1, 3, 5 or 7. DNA sequences encoding secreted forms of human VEGF are preferred. Thus, DNA sequences of SEQ ID No. 1 and 3 are particularly preferred.

30   When VEGF protein is used, VEGF protein having the amino acid sequence of SEQ ID No. 2, 4, 6 or 8 is preferred. Secreted forms of VEGF are preferred. Thus, VEGF-121 (SEQ ID No. 2) and VEGF-165 (SEQ ID No. 4) are particularly preferred.

However, the VEGF DNA and proteins suitable for the practice
of the invention are not limited to those specific sequences.
Rather, the invention also provides for the use of other
closely related DNA and protein sequences.

5   DNA sequences of the invention may be related to that of SEQ
ID No. 1, 3, 5 or 7 in a number of ways. For example, DNA
sequences suitable for the practice of the invention may be
degenerate sequences that encode the same protein, the
protein of SEQ ID No. 2, 4, 6 or 8.

10   Alternatively, DNA sequences of the invention may be
substantially homologous to that of SEQ ID No. 1, 3, 5 or 7,
and encode a protein that differs in amino acid sequence from
that of SEQ ID No. 2, 4, 6 or 8 but encodes a protein having
VEGF activity. Typically, DNA sequences of the invention have
15   at least 70%, at least 80%, at least 90%, at least 95%, or at
least 99% sequence homology to the sequence of SEQ ID No. 1,
3, 5 or 7.

DNA sequences of the invention may, for example, be genomic
DNAs or cDNAs, or hybrids between genomic DNA and cDNA, or
20   they may be synthetic or semi-synthetic. They may originate
from any species, though DNAs encoding human VEGF are
preferred. Genomic DNAs encoding the proteins of SEQ ID. No.
2, 4, 6 and 8 are particularly preferred.

DNA sequences of the invention may be single-stranded or
25   double-stranded.

DNA sequences of the invention may differ from the sequence
shown in SEQ ID No. 1, 3, 5 or 7 by the deletion, insertion
or substitution of one or more nucleotides, provided that
they encode a protein having VEGF activity. Similarly, they
30   may be truncated with respect to SEQ ID No. 1, 3, 5 or 7 or
extended by one or more nucleotides provided that they encode
a protein having VEGF activity.

RNA sequences are also suitable for the practice of the
invention. In particular, the invention provides for the use
of the RNA sequence corresponding to that of SEQ ID No. 1,
3, 5 or 7 which is a preferred RNA sequence. The invention
5   also provides for the use of RNA sequences that are related
to this sequence of in any of the ways described above for
DNA sequences. RNA sequences for the invention may be single-
stranded or double-stranded. RNAs of the invention may be of
any origin. For example, they may originate from any
10   species, although RNAs encoding human VEGF, especially human
VEGF having the sequence shown in SEQ ID. No. 2, 4, 6 or 8
are preferred. Synthetic DNA s may also be used, as may
semi-synthetic RNAs. Further, DNA transcribed form bacterial
plasmids *in vivo* or *in vitro* may be used.
15

It will be appreciated by those of skill in the art that, in
RNA sequences suitable for the practice of the invention, the
T residues will be replaced by U.

VEGF proteins of the invention are encoded by DNA or RNA
20   sequences of the invention as defined above.   Preferred
proteins of the invention are the proteins of SEQ ID No. 2,
4, 6 and 8 though the invention also provides for the use of
other proteins having closely related sequences that differ
from those of SEQ ID No. 2, 4, 6 or 8 but have VEGF activity.

25   VEGF activity, according to the invention, is the ability
completely or partially to inhibit or prevent intimal
hyperplasia of a blood vessel, particularly an artery.
Proteins of the invention that differ slightly in sequence
from naturally occurring VEGF, as described above, retain
30   this property, although not necessarily to the same extent as
VEGF. Similarly, such proteins may exhibit stronger VEGF
activity than naturally occurring VEGF. VEGF proteins
suitable for the practice of the invention also typically
exhibit one or more of th biological properties of VEGF that
35   are already known in the art, such as the ability to promote

the proliferation of arterial EC *in vitro* and/or *in vivo*.

VEGF proteins suitable for the practice of the invention may therefore be substantially homologous to the VEGF of SEQ ID No. 2, 4, 6 or 8 typically at least 70%, at least 80%, at

5 least 90%, at least 95%, or at least 99% homologous.

VEGF proteins suitable for the practice of the invention may differ from the sequence shown in SEQ ID No. 2, 4, 6 or 8 by the deletion, insertion or substitution of one or more amino acids, provided that they have VEGF activity. Similarly, they

10 may be truncated by one or more amino acids with respect to SEQ ID No. 2, 4, 6 or 8 or extended with respect to SEQ ID No. 2, 4, 6 or 8 by one or more amino acids, provided that they have VEGF activity. In respect of substitutions, conservative substitutions are preferred. Typically,

15 conservative substitutions are substitutions in which the substituted amino acid is of a similar nature to the one present in naturally occurring VEGF, for example in terms of charge and/or size and/or polarity and/or hydrophobicity. Similarly, conservative substitutions typically have little

20 or no effect on the VEGF activity of the protein.

VEGF proteins of the invention that differ in sequence from naturally occurring VEGF may be engineered to differ in activity from naturally occurring VEGF. For example, they may be engineered to have stronger VEGF activity. Such

25 manipulations will typically be carried out at the nucleic acid level using recombinant techniques known in the art.

In the practice of the invention, VEGF or a nucleic acid encoding VEGF may be delivered to an artery in any suitable form.

30 It is preferred to deliver nucleic acids encoding VEGF, rather than VEGF protein, thereby to effect gene therapy of the hyperplasia to be treated. These nucleic acids may b

delivered in a "naked" form unassociated with a vector, or by means of a gene therapy vector. It is preferred to deliver them by means of a gene therapy vector.

Any suitable gene therapy vector may be used. In particular,
5   viral or non-viral vectors may be used.

Suitable viral vectors include adenoviruses, retroviruses, pseudotyped retroviruses, herpesviruses, vaccinia viruses and baculoviruses.

Suitable non-viral vectors include oligonucleotides,
10   plasmids, liposomes, cationic liposomes, pH sensitive liposomes, liposome-protein complexes, immunoliposomes, liposome-protein-polylysine derivatives, water-oil emulsions, polyethylene imines and dendrimers.

Where appropriate, two or more types of vector can be used
15   together. For example, a plasmid vector may be used in conjunction with liposomes.

Preferred vectors include Moloney murine leukaemia virus (MMLV)-derived retroviruses, pseudotyped vesicular stomatitis virus protein-G (VSV-G)-containing retroviruses,
20   adenoviruses, plasmids and plasmid/liposome complexes.

Suitable liposomes include, for example, those comprising the positively charged lipid (N[1-(2,3-dioleyloxy)propyl]-N,N,N-triethylammonium (DOTMA), those comprising dioleoylphosphatidylethanolamine (DOPE), and those comprising
25   3β[N-(n',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol).

Viral vectors of the invention are preferably disabled, e.g. replication-deficient. That is, they lack one or more functional genes required for their replication, which
30   prevents their uncontrolled proliferation *in vivo* and avoids

undesirable side effects of viral infection. Preferably, all
of the viral genome is removed except for the minimum genomic
elements required to package the viral genome incorporating
the VEGF nucleic acid into the viral coat or capsid. For

5   example, it is desirable to delete all the viral genome
except the Long Terminal Repeats (LTRs) and a packaging
signal. In the case of adenoviruses, deletions are typically
made in the E1 region and optionally in one or more of the
E2, E3 and/or E4 regions.

10   Viruses of the invention may be disabled by any suitable
technique. For example, genomic deletions may involve
complete removal of genes required for replication, or only
partial removal. Complete removal is preferred. In general,
preferred deletions are of genes required for early

15   transcription of viral genes.

Replication-competent self-limiting or self-destructing viral
vectors may also be used.

In general, the VEGF nucleic acids of the invention will be
comprised within an expression construct that ensures their

20   expression *in vivo* after they have been delivered to the
artery, preferably by a vector as defined above. Such
constructs typically comprise, a promoter capable of
directing the expression of the VEGF nucleic acid of the
invention, and optionally a regulator of the promoter, a

25   translational start codon, and, operably linked to the
promoter, a VEGF nucleic acid according to the invention.
Preferably, these components are arranged in a 5'-3'
orientation.

The construct may also comprise any other suitable

30   components. For example, the construct may comprise a nucleic
acid encoding a signal sequence, so positioned in such a
position relative to the VEGF nucleic acid such that, when it
is translated, it is capable of directing the expressed VEGF

protein to a given cell type or cell compartment. Any such
signal sequence will typically be positioned immediately 3'
or immediately 5' to the VEGF nucleic acid, such that the
signal sequence and VEGF protein are translated as a single
5   fusion protein, with the signal sequence at the C- or N-
terminus.

The construct may also comprise an enhancer which enhances
the degree of expression provided by the promoter. Any
enhancer which enhances the expression provided by the
10   selected promoter may be used. For example, in the case of
the CMV early gene promoter, the CMV early gene enhancer may
be used.

Optionally, the construct may comprise a transcriptional
terminator 3' to the VEGF nucleic acid. Any suitable
15   terminator may be used.

Optionally, the construct may comprise a polyadenylation
signal operably linked 3' to the VEGF nucleic acid.

Optionally, the construct may comprise one or more selectable
20   marker genes, e.g. antibiotic resistance genes, to allow
selection of transformed cells in culture. For example,
cells may be selected for antibiotic resistance in order.

Optionally, the construct may comprise one or more introns,
or other non-coding sequences, for example 3' or 5' to the
25   VEGF nucleic acid.

Any suitable promoter may be used to control the expression
of the nucleic acid of the invention. In general, it is
preferred to use a viral promoter or a promoter adapted to
function in the species of the subject to be treated. Thus,
30   in the case of a human subject, it is preferred to use viral
promoters, especially promoters derived from viruses that
infect humans, or promoters derived from human genes.

Optionally, a promoter may be used in combination with any
suitable enhancer.

Desirably, a "strong" promoter is used, i.e. one that secures
high levels of expression of the VEGF protein of the

5   invention. Promoters that achieve overexpression of the VEGF
protein are desirable. Preferred promoters include the
cytomegalovirus (CMV) promoter, optionally in combination
with the CMV enhancer; the human β-actin promoter; the simian
virus 40 (SV40) early gene promoter; the Rous sarcoma virus

10   (RSV) promoter; and the retroviral long terminal repeat (LTR)
promoter.

Promoters, and other construct components, are operably
linked to the VEGF nucleic acid of the invention. Thus, they
are positioned in order that they may exert their effect on

15   expression of the VEGF nucleic acid. For example, in the case
of a promoter, the promoter is positioned relative to the
VEGF nucleic acid such that it is able to direct expression
of the VEGF nucleic acid. Desirably, construct components are
positioned to allow them to exert their maximum effect on

20   expression.

Nucleic acids of the invention, or constructs of the
invention, may be incorporated into viral genomes by any
suitable means known in the art. Viral genomes may then be
packaged into viral coats or capsids by any suitable

25   procedure. In particular, any suitable packaging cell line
may be used to generate viral vectors of the invention. These
packaging lines complement the replication-deficient viral
genomes of the invention, as they include, typically
incorporated into their genomes, the genes which have been

30   deleted from the replication-deficient genome. Thus, the use
of packaging lines allows viral vectors of the invention to
be generated in culture.

Suitable packaging lines include derivatives of PA317 cells,

Ψ-2 cells, CRE cells, CRIP cells, E-86-GP cells, Fly cells,
line 293 cells and 293GP cells.

In the case of non-viral vectors, nucleic acid may be
incorporated into the non-viral vectors by any suitable means
5   known in the art.

As desired, vectors, especially viral vectors, may be
selected to achieve integration of the nucleic acid of the
invention, or of a construct of the invention, into the
genome of the cells of the subject to be treated, or to leave
10   the nucleic acid or construct free in the cytoplasm.
Integrative vectors are preferred.

VEGF proteins of the invention or VEGF nucleic acids of the
invention, preferably associated with a viral or non-viral
vector, as described above, may be administered to arteries
15   in any suitable manner in order to effect treatment of
hyperplasia. For example, VEGF or a nucleic acid encoding
VEGF may be administered to the exterior wall of the blood
vessel, e.g. artery, or to the blood vessel endothelium, e.g.
the arterial endothelium, for example via the lumen. Local
20   gene transfer is likely to be advantageous over the
administration of recombinant VEGF protein since infused
compounds are rapidly flushed away by blood flow and short
half-life in blood.

Once delivered, VEGF nucleic acids of the invention are
25   expressed to produce VEGF proteins, which in turn effect
treatment or prevention of intimal hyperplasia. Expression
may take place in any cell type or types in the blood vessel,
e.g. arterial, wall.

Preferably, expression occurs in such a location that the
30   expressed VEGF is able to reach the endoth lium of the blood
vessel, e.g. artery. For example, expression may occur in
the smooth muscle cells and/or in th endothelium. Most

preferably, expression takes place at least in the
endothelium of the blood vessel, e.g. artery.

For example, VEGF protein or nucleic acids, may be delivered
to the outside of the blood vessel, e.g. artery, by direct
injection around the site of the hyperplasia to be treated or
prevented, or by injection into the lumen of the blood
vessel, e.g. artery.

More preferably, the VEGF proteins of the invention or VEGF
nucleic acids of the invention, typically associated with a
viral or non-viral vector, as described above, are delivered
by means of an implant placed externally to the blood vessel,
e.g. artery, in proximity to the site of the hyperplasia to
be treated. Such an implant contains the VEGF protein or
nucleic acid or the vector and provides a reservoir of the
VEGF protein or nucleic acid or vector. The VEGF protein or
nucleic acid (preferably in association with a vector) may be
introduced into the implant before or after the implant is
introduced into the subject to be treated. For example, the
implant may be fitted in the vicinity of the blood vessel,
with the VEGF protein or nucleic acid being introduced into
the implant, e.g. by injection, subsequently.

Preferably, the implant is placed in direct contact with the
blood vessel, e.g. artery. This is especially preferred when
retroviral vectors are used to deliver VEGF nucleic acids of
the invention, as the physical distortion of the blood vessel
may induce smooth muscle cell proliferation, which increases
the efficiency of gene transfer by retroviral vectors. This
proliferation, like the proliferation induced by the
hyperplasia itself, is overcome or at least ameliorated, by
the delivery of VEGF protein or nucleic acid according to the
invention. Similarly, it is preferred for the implant to be
in contact with the artery when other vectors that xhibit
increased efficiency of gene transfer when their target cells
are dividing are employed. For example, cell proliferation

may also enhance gene transfer efficiency with plasmid/liposome complex s.

Such implants may be in any suitable form. Preferably, the implant is in the form of a collar which surrounds, partially or completely, preferably completely, the artery, at or near the site of the hyperplasia to be treated or prevented.

Intravascular procedures, e.g. using balloon catheterization or high pressure fluid may lead to endothelial damage or denudation. In the preferred embodiment of the invention discussed above, extravascular gene delivery is used. Transfected genes are preferably applied via a silastic or biodegradable implant, preferably a collar placed next to, preferably around, the outside of the blood vessel, and the endothelium does not suffer significant damage; preferably no damage at all is suffered. This is a major advantage of this form of delivery.

When, according to the invention, vectors are applied directly on the adventitial surface of a blood vessel within a collar, close contact with the adventitia is maintained. In rabbit arteries, a collar alone typically leads to the formation of a neointima within 7-14 days after the operation and a similar situation can be expected in human patients.

The collar also maintains a high concentration of vector at the adventitial surface.

Implants, preferably collars, may be made of any suitable material. Silastic implants, i.e. implants comprising silicone rubbers, are one preferred alternative. Most preferred are biodegradable implants. Any suitable biodegradable material may be used.

Thus, in a preferred embodiment, the implant is in the form of a biodegradable or silastic collar, containing a VEGF

nucleic acid of the invention, typically comprised within a vector as defined above and placed around an artery at or near the site of an intimal hyperplasia to be treated or prevented.

5   Within the implant, e.g. collar, the VEGF protein or nucleic acid may be contained in any way. Preferably, the structure of the implant, e.g. collar, is such that the VEGF protein or nucleic acid is held in direct contact with the blood vessel wall. Thus, in one embodiment, the structure of the implant

10 leaves a space between the blood vessel wall and the wall of the implant. In the case of collar, the implant thus forms a hollow container around the blood vessel. Into this space, VEGF nucleic acids or proteins can be introduced, such that they are in contact with the blood vessel wall. Preferably,

15 the extremities of the implant are in contact with the blood vessel wall, thus preventing the escape of the VEGF nucleic acid or protein. Preferably, the outer wall of the collar is impermeable, or substantially impermeable, to the VEGF nucleic acid or protein, thus preventing, or at least

20 limiting, its escape into the surrounding tissue and ensuring its delivery to the blood vessel. A preferred implant of this type, in the form of a collar is shown in Figures 7 and 8.

Optionally, the space containing the VEGF nucleic acid or

25 protein may be separated from the wall of the blood vessel by one or more layers of material permeable or semi-permeable to the VEGF or nucleic acid. This may be desirable if gradual delivery is intended and is desired to limit the rate at which VEGF protein or nucleic acid is delivered to the blood

30 vessel wall.

Optionally, the implant, e.g. collar, may be designed to act as an asmatic pump.

Optionally, the VEGF may be contained within a medium within

the collar, e.g. a solid or gel medium. This way help to
prevent the VEGF protein or nucleic acid escaping into the
tissue. In this case, the outer wall of the collar may not
need to be in contact with the blood vessel of the extremity
5   of the implant.

Alternatively, the VEGF nucleic acid or protein may be coated
onto the surface of the implant which is in contact with the
blood vessel in use. Alternatively, the VEGF nucleic acid or
protein may be dispersed throughout the structure of the
10   implant.

Some advantages of the use of implants in this way,
especially collars, are: (i) they provide a delivery
reservoir, allowing for sustained delivery; (ii) no
intralumenal manipulations are required and the arterial
15   endothelium remains intact; and (iii) the distortion (e.g.
constriction in the case of a collar) created by the implant
may enhance the efficiency of gene delivery, as explained
above.

The proteins or nucleic acids of the invention may be applied
20   to the treatment or prevention of intimal hyperplasia arising
from any clinical circumstances. For example, it is possible
to treat hyperplasia arising after any type of surgical
procedure, including angioplasty, for example balloon
angioplasty; bypass surgery, such as coronary bypass surgery
25   in which a vein is anastomosed to an artery; other
anastomosis procedures, for example anastomosis in the legs;
and endarteriectomy, for example carotid artery
endarteriectomy. It is also possible to treat intimal
hyperplasia associated with arterial damage or hypertension,
30   for example pulmonary artery hypertension.

The invention provides for treatment of intimal hyperplasia
in any type of blood vessel, e.g. in an artery or vein,
preferably an artery.

According to the invention, it is possible to treat or ameliorate established intimal hyperplasia or to prevent intimal hyperplasia from arising. Similarly, it is possible to diminish the likelihood of intimal hyperplasia arising, or

5   to diminish the severity of established intimal hyperlasia or hyprplasia that is likely to arise. Treatment according to the invention may take place before, during, or after a surgical procedure, for example in order to reduce the chance of hyperplasia arising after the procedure.

10   The proteins or nucleic acids of the invention are preferably delivered in the form of a pharmaceutical formulation comprising a pharmaceutically acceptable carrier. Any suitable pharmaceutical formulation may be used.

For example, suitable formulations may include aqueous and

15   non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats, bactericidal antibiotics and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending

20   agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for

25   injections, immediately prior to use.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in questions. Of

30   the possible formulations, sterile pyrogen-free aqueous and non-aqueous solutions ar preferred.

The proteins, nucleic acids and vectors of the invention may

be d livered in any suitable dosage, and using any suitable
.dosage regime. Persons of skill in the art will appreciate
that the dosage amount and regime may be adapted to ensure
optimal treatment of the particular condition to be treated,

5   depending on numerous factors. Some such factors may be the
age, sex and clinical condition of the subject to be treated.

For the delivery of naked nucleic acids encoding VEGF or
constructs comprising such nucleic acids, typical doses are
from 0.1 - 5000μg, for example 50 - 2000μg, such as 50 - 100

10   μg, 100 - 500μg and 500-2000 μg per dose.

For the delivery of VEGF protein, suitable doses include
doses of from 1 to 1000μg for example from 1 to 10μg, from 10
to 100μg, from 100 to 500μg and from 500 to 1000μg.

The dosage used for the delivery of VEGF nucleic acids by

15   means of viral or non-viral vectors will depend on many
factors, including the efficiency with which the vectors
deliver VEGF nucleic acids to cells, and the efficiency with
which the VEGF nucleic acids are expressed in the cells.

For example, viral vectors may be delivered in doses of from

20 . 10^4 to 10^{14} cfu or pfu/ml, for example 10^4 to 10^6, 10^6 to 10^8,
10^8 to 10^{10}, 10^{10} to 10^{12} or 10^{12} to 10^{14} cfu pfu/ml. Doses in
the region of 10^5 to 10^9 cfu or pfu/ml are preferred. The
term pfu (plaque forming unit) applies to certain viruses,
including adenoviruses, and corresponds to the infectivity of

25   a virus solution, and is determined by infection of an
appropriate cell culture, and measurement, generally after 48
hours, of the number of plaques of infected cells. The term
cfu (colony forming unit) applies to other viruses, including
retroviruses, and is determined by means known in the art

30   generally following 14 days incubation with a selectable
marker. The techniques for determining the cfu or pfu titre
of a viral solution are well known in the art.

For retroviruses, dosages in the r gion of 10^5 to 10^6 pfu/ml
are particularly preferred. For pseudotyped retroviruses,
dosages in the region of 10^7 pfu/ml are particularly
preferred. For adenoviruses, dosages in the region of 10^9

5   pfu/ml are particularly preferred.

Similarly, such doses may be included within implants of the
invention for gradual delivery.

VEGF nucleic acids associated with non-viral vectors may also
be delivered in any suitable dosage, by any means of

10   administration, as described above, or gradually from an
implant. Suitable doses are typically from 0.1 to 1000μg of
nucleic acid, for example 1 to 100 μg, 100 to 500μg or 500 to
1000 μg, 1000 to 2000 μg, 2000 to 3000 μg or 3000 to 5000μg.
Preferred doses are in the region of 5 to 50μg, for example

15   10 to 20μg.

Dosage schedules will also vary according to, for example,
the route of administration, the species of the recipient and
the condition of the recipient. However, single doses and
multiple doses spread over periods of days, weeks or months

20   are envisaged. Also, as explained above, the delivery of
VEGF proteins and nucleic acids may be effected by means of
an implant suitable for fitting around a blood vessel,
preferably an artery; preferably, the implant is in the form
of a collar. Such an implant will effect gradual delivery.

25   For example, delivery may take place over a period of hours,
days, weeks or months.

Proteins and nucleic acids of the invention may be
administered by any form of administration, for example
topical, cutaneous, parenteral, intramuscular, subcutaneous

30   or transdermal administration, or by direct injection into
the bloodstream, direct inj ction into or around the arterial
wall or by direct application to mucosal tissues.

Preferably, administration is by means of an implant, as described above.

The proteins, nucleic acids and vectors of the invention may be used to treat intimal hyperplasia in any mammal. Treatment

5   of human patients is preferred.

The invention also provides kits for the treatment or prevention of intimal hyperplasia. These kits comprise (i) VEGF protein or nucleic acid of the invention, preferably in association with a vector, as defined above; and (ii) an

10   implant of the invention in the form of a collar into which the VEGF protein or nucleic acid may be introduced. Preferably, the VEGF nucleic acid or protein is provided in the form of a pharmaceutical formulation comprising a pharmaceutically acceptable carrier as defined above.

15   Components (i) and (ii) may be packaged in any suitable way. Other components known in the art may also be included, for example standard reagents and/or solutions and/or equipment.

The invention also provides methods of treating or preventing intimal hyperplasia comprising administering to a patient in

20   need of such treatment, an effective non-toxic amount of a VEGF protein, nucleic acid or vector of the invention. Such treatment is effected in the manner described herein.

The implants of the invention, especially implants in the form of collars, as defined above, can also be used for the

25   delivery of agents other than VEGF to blood vessels, e.g. arteries. Any suitable agent may be delivered in this way, to achieve any desired therapeutic goal.

The inventors have observed that plasmid/liposome complexes, MMLV retroviruses, VSV-G retroviruses and adenoviruses lead

30   to expression in collared arteries. Gene transfer efficiency was highest with adenoviruses and pseudotyped VSV-G retroviruses also produced a relatively high transfection

efficiency. The utility of the replication-deficient VSV-G retroviruses in arterial gene transfer has not be n previously demonstrated. Expression was seen in some endothelial cells of the adenovirus-transfected arteries.

5 Since penetration from the adventitia to the intima had occurred, these results raise the general possibility of altering endothelial function in human disease by extralumenal gene transfer using genes other than VEGF. This may also be useful for the expression in the adventitia and

10 outer media of diffusible or secreted gene products which then act elsewhere in the arterial wall. Preferably, such delivery effects treatment of intimal hyperplasia, as defined above, although it may also effect additional or alternative therapeutic goals.

15 Preferred therapeutic agents for delivery in this manner include proteins other than VEGF that stimulate nitric oxide (NO) production in the arterial wall. The delivery of (NO) synthases, especially inducible NO synthase (iNOS) to effect treatment or prevention of intimal hyperplasia is

20 particularly preferred. Other preferred therapeutic agents include synthetic agonists which activate the endothelial VEGF receptor. These are preferably delivered by the same route, i.e. via an implant as defined herein, though they could be delivered systemically.

25 Preferably, the therapeutic agent will be in the form of nucleic acid encoding a pharmaceutically active polypeptide or protein. More preferably, this nucleic acid will be comprised within a construct, as defined above. Still more preferably, the nucleic acid or construct will be delivered

30 to the artery by means of a vector as defined above, for example a viral or non-viral vector as defined above.

Thus, extra-arterial gene transfer can be used for the delivery of genetic material into the wall of blood vessels, preferably arteries. From the xamples, it can be seen that

changes in medial SMC and even endothelium change can be achieved from the adventitial side, allowing the development of new methods for the treatment of blood vessel, e.g. arterial, disease.

5 Accordingly, the invention provides the use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of a blood vessel, of NOS, (optionally iNOS), or a nucleic acid encoding NOS, (optionally iNOS); wherein the NOS protein or nucleic acid is provided in an
10 implant, preferably a collar, as defined above for VEGF.

The invention also provides kits for the treatment or prevention of intimal hyperplasia of a blood vessel comprising (i) NOS (optionally iNOS) protein or nucleic acid; and (ii) an implant of the invention. These kits are as
15 described above for VEGF.

The invention also provides a method of treating intimal hyperplasia of a blood vessel comprising implanting an implant of the invention comprising NOS protein or nucleic acid in the vicinity of the hyperplasia to be treated or
20 prevented, thereby to effect delivery of NOS protein or nucleic acid.

NOS nucleic acid is preferably associated with a vector, as described above for VEGF. Treatment is carried out as described above for VEGF, and dosages and pharmaceutical
25 formulations are also as described above for VEGF.

The invention also provides implants of the invention which comprise NOS, (optionally iNOS), protein or nucleic acid.

The following Examples illustrate the invention.

EXAMPLES

30 **Exampl 1**

VEGF gene transfer inhibits SMC prolif ration, probably acting via th NO pathway.

The effect of endothelial cell (EC)-specific vascular endothelial growth factor (VEGF) gene transfer on the
5 thickening of the intima was studied using a silicone collar inserted around carotid arteries which acted both as the agent that caused intimal smooth muscle cell growth and as a reservoir for the gene and vector. The model preserved EC integrity and permitted direct extravascular gene transfer
10 without any intravascular manipulation. Compared to β-galactosidase (lacZ)-transfected control arteries, plasmid/liposome-mediated VEGF gene transfer significantly reduced intimal thickening one week after the gene transfer. Administration to the experimental animals of the NO
15 synthesis inhibitor L-NAME abolished the difference in intimal thickening between VEGF and LacZ-transfected arteries. Furthermore, VEGF caused NO release from human umbilical vein ECs *in vitro*. It is concluded that VEGF gene transfer reduces intimal thickening and is useful for the
20 treatment of restenosis. These results further suggest that VEGF may reduce smooth muscle cell proliferation via a novel mechanism involving VEGF-induced NO production.

Forty 3-4 months old New Zealand White rabbits were used for the studies with an expression vector containing cDNA for the
25 secreted form of VEGF (Breier *et al*: Development 1992; 114: 521-532). 25 μg pCMV-VEGF-164 (also known as VEGF-165) plasmid was complexed with 25μg Lipofectin (BRL) and diluted to 500μl with Ringer solution. Control arteries were treated in the same way with lacZ/liposome complexes. It was found
30 that, as compared with lacZ-transduced arteries, VEGF gene transfer significantly reduced intimal thickening one week after the operation (intima/media ratio 0.3 vs 1.1,<0.05, respectively, Figure 1A). The effect was reduced after two weeks which is probably due to the fact that the
35 plasmid/liposome-mediated gene transfer typically only

induces temporary expression of the transfected gene with
maximal protein expression between 2-3 days after the gene
transfer (Nabel et al Annu. Rev. Physiol. 1994; 56: 741-761).
Immunohistochemical analysis of the arteries showed that

5   intimal thickening was almost exclusively composed of SMC
(Figure 2A/B). Endothelial layer was present in all stadred
segments. No adverse effects or inflammation were detected
in the transfected arteries (Figure 2). Expression of the
transfected VEGF was confirmed by RT-PCR using primers

10   specific for the transgene (Figure 1B) and by in situ
hybridization. Most of the VEGF (Figure 2) and lacZ
expression occurred in the adventitia and outer media in
fibroblasts and SMCs. Adventitial neovascularization was
seen in three of the VEGF-transfected arteries 14d after the

15   gene transfer (Figure 2). No neovascularization was detected
in lacZ-transfected arteries. We have shown previously that
lacZ-plasmid/liposome gene transfer using the collar model
leads to a local gene transfer in 0.05% of arterial cells.
In spite of the low gene transfer efficiency, the secreted

20   form of VEGF produced inside the collar leads to biological
effects in the local arterial microenvironment, as indicated
by the presence of neuvascularisation in three VEGF-
transfected arteries 14 days after gene transfer (Figure 2F).
As in acute hypoxia, secreted VEGF is believed to reach the

25   EC by diffusion and bind to VEGF receptors on EC.

It was hypothesized that the inhibitory effects of VEGF on
intimal thickening were due to either a direct or indirect
VEGF-induced EC-derived factor or activity that could, in
turn, inhibit SMC proliferation. In particular, it was

30   hypothesized that the effects of VEGF on intimal thickening
were mediated through the NO pathway. This hypothesis was
tested in a subset of New Zealand White rabbits (n=8) by
giving the animals NO synthase inhibitor L-NAME during the
gene transfer experiments. It was found that L-NAME

35   abolished the difference in intimal thickening between VEGF-
and lacZ-transfected arteries (Figure 3A). The main target

cells for VEGF in the art rial wall are EC. The only other cell types possessing VEGF receptors are monocytes but as judged from immunocytochemistry with specific antibodies, monocytes are absent from the collared carotid arteries under
5 these conditions (Figure 2).

The results shown in Figure 3 were consistent with VEGF-induced inhibition of intimal thickening through the stimulation of NO production. We therefore examined whether VEGF could directly stimulate NO production in cultures of
10 EC. VEGF induced tyrosine phosphorylation of a major 205 kDa protein corresponding to the VEGF receptor within the concentration range 1-25ng/ml (Figures 4A and B). As shown in Figures 4C and D, addition of VEGF to cultured human umbilical vein endothelial cells (HUVEC) caused a time- and
15 concentration-dependent increase in NO production as monitored by measurement of nitrite levels. The effect of VEGF on NO production was seen as early as 30 seconds after the addition of VEGF, reached a maximum after 5 min and was sustained for up to 2 hours. The half-maximal effect of VEGF
20 was obtained at 5ng/ml. VEGF-induced phosphorylation (Figure 4B) and NO production (Figure 4D) were completely abolished in the presence of 100μM L-NAME (Figure 3C). Thus, it is likely that VEGF gene transfer stimulates NO production in EC in the transfected arteries and limits SMC proliferation at
25 least partially via an NO-mediated mechanism. The findings are compatible with the previous observations that transfection of arteries with endothelial NO synthase cDNA reduces intimal thickening (Von der Leyon et al: PNAS 1995; 92: 1137-1141).

30 VEGF is an important EC-specific mitogen during embryonal development and has been shown to act as EC "survival factor" in retinal arteries. Recently, other forms of VEGF have also been identified (VEGF-B (Olofsson et al: PNAS 1996; 93: 2576-2581); and VEGF-C (Joukov et al: EMBO J. 1996; 15: 290-298)).
35 Asahara et al (Circulation 1995; 91: 2793-2801) have

demonstrated that local delivery of VEGF recombinant protein into balloon denuded rat carotid arteries significantly enhanced reendothelialization and consequently reduced intimal thickening. Previous studies in arterial denudation
5   models have also established an inverse relationship between EC integrity and SMC proliferation; and that effective regeneration of EC is one of the most potent inhibitors of SMC proliferation (Callow et al supra; Asahara et al, supra). Callow et al (supra) and Asahara et al (supra) concluded that
10 administration of VEGF protein stimulated EC proliferation in denuded arteries, but the actual mechanisms involved in the inhibition of intimal thickening were not studied. in the collared carotid artery, intimal thickening is stimulated in the presence of an anatomically intact endothelium.
15 Therefore, it is unlikely that the inhibitory effect of arterial VEGF gene transfer on intimal thickening reported here is due to VEGF-stimulated reendothelialization. According to these results VEGF can directly induce NO production in HUVEC such that this is one mechanism through
20 which VEGF can inhibit intimal thickening. VEGF may also stimulate the production of other factors which can negatively regulate SMC proliferation including TGF-β or prostacyclin.

Example 1.1

25 **VEGF gene transfer reduces intimal thickening in rabbit carotid arteries 7 days after the gene transfer (Figure 1). VEGF transgene mRNA expression in arteries transfected with VEGF (lane 1) or lacZ (lane 2) plasmid/liposomes as analyzed using RT-PCT (Figure 1B).**

30 **Gene Transfer.** Intimal thickening was induced in the carotid arteries of thirty-two New Zealand White rabbits by inserting an inert silicone collar around the arteries under a general anesthesia (Booth et al: Atherosclerosis 1989; 76: 257-268). Gene transfer was done five days after positioning of the

collar by gently opening the collar under anesthesia and
injecting 500ul plasmid/liposome complexes into the collar
(i.e. on the adventitial surface of the artery). No
intravascular manipulations were involved in any steps of the

5   studies. Plasmid/liposome complexes were made as follows.
Twenty-five μg pCMV5-VEGF-164 plasmid (containing mouse VEGF
cDNA (Breier *et al*, *supra*, nucleotides 1-583) was complexed
with 25ul Lipofectin (BRL) while diluted to 500ul with Ringer
solution. Complexes were kept at room temperature at least

10   15min before the gene transfer. It was determined previously
that at the concentration used in the present study
plasmid/Lipofectin complexes were not toxic to rabbit aortic
EC *in vitro*. Control arteries were transfected with a
similar plasmid/liposome complex containing *E.coli* lacZ cDNA

15   (Kalnins *et al*, *supra*) (nucleotides 1-3100) expression
plasmid. Plasmids used for the studies were isolated from
E.coli cultures (DH5α) using Qiagen Mega columns and purified
using three phenol/chloroform extractions and one ethanol
precipitation (Ausubel FM, Brent R, Kingston RE, Moore DD,

20   Seidman JG, Smith JA, Struhl K, eds. Current Protocols in
Molecular Biology. New York, NY: Greene Publishing
Associates and John Wiley & Sons; 1991;4.2.3-4.2.4) were
adjusted to 1μg/μl and analyzed to be free of any
microbiological or endotoxin contamination (Limulus assay,

25   detection limit 0.2ng). Animals were sacrificed 3 (n=8), 7
(n-12) and 14 (n=12) days after the gene transfer operation,
arteries were carefully removed and divided into three equal
portions: the proximal third was immersion-fixed in 4%
paraformaldehyde/PBS for 15 min and embedded in OCT compound

30   (Miles Scientific) (Ylä-Herttuala *et al*: J. Clin. Invest.
1995; 95: 2692-2698). The middle third was fixed as above
for 4h, rinsed in 15% sucrose for 48h and embedded in
paraffin. The distal third was directly embedded in OCT
compound and frozen in liquid nitrogen. In four arteries the

35   distal third was used for mRNA isolation and RT-PCR (see
below) Ten randomly selected sections from the middle portion
were used for the determination of intima/media thickness

ratio (Ylä-Herttuala *et al*: Arteriosclerosis 1986; 6; 230-236) by two independent observers without knowledge of the origin of the samples. Mean values of the two independent measurements were used to calculate the results (mean ± SD).

5   Differences in the intima/media thickness ratios between the groups were analyzed by ANOVA, followed by modified t-test (* p<0.05).

RT-PCR. Distal portions of VEGF (n=2) and lacZ (n=2) transfected arteries collected 7 days after the gene transfer

10 were used for mRNA isolation (Micro-FastTrack, Invitrogen) and were reverse-transcribed to the first strand cDNA using AMLV reverse transcriptase (5U per reaction, Boehringer) using random hexamer primers (cDNA cycle Kit, Invitrogen) as described (Hiltunen *et al*: Circulation 1995; 92:3297-3303).

15 A thirty-five cycle PCR was performed with Taq polymerase (Boehringer) and primers specific for the transfected pCMV5-VEGF-164 construct (5'-primer: TCG ATC CAT GAA CTT TCT GC (SEQ ID. No. 9) 3'-primer: TCC GTT TAA CTC AAG CTG CC (SEQ ID. No. 10); PCR cycle parameters: 1min 90°C, 1min 60°C, 1min

20 72°C, except for the last cycle 5min). Amplified fragment with an expected length (547nt) was seen in the VEGF-transfected arteries. DNA size markers (1kb ladder, BRL) are shown on both sides of the gel.

Example 1.2

25 **Gene transfer was carried out as described in Example 1 and representative micrographs of the characteristics of rabbit carotid arteries 7d after VEGF or lacZ gene transfer using the collar model are shown in Figure 2.**

(A-G) immunostainings of the transfected arteries: (A)

30 Control artery transfected with lacZ-plasmid/liposomes (SMC-specific MAb HHF-35, 1:500 dilution, Enzo Diagnostics) showing typical intimal thickening; (B) Artery transfected with VEGF plasmid/liposomes (SMC-specific MAb HHF-35) where

only limited intimal thickening is s en; (C) Endothelium was
present in all studied vascular segments (serial section to
A, EC-specific MAb CD31, dilution 1:50, DAKO); (D)
Endothelial staining (serial section of B, MAb CD31); (E) No
5   evidence of inflammation was detected in VEGF-and lacZ-
transfected arteries (Macrophage-specific MAb RAM-11 dilution
1;500, DKO); (F) Neovascularization in the adventitia of
VEGF-transfected artery 14 days after gene transfer
(Hematoxylin-eosin staining); (G) Non-immune control for the
10   immunostainings (first antibody omitted).

The avidin-biotin horseradish peroxidase system (Vector
Elite, Vector Labs) was used for the immunostainings (Ylä-
Herttuala *et al*: PNAS 1990; 87: 6959-6963). Controls for the
immunostainings included incubations with irrelevant class-
15   and species-matched immunoglobulins and incubations where the
primary antibodies were omitted. *In situ* hybridizations were
done using an anti-sense VEGF riboprobe (583nt) synthesized
from pBluescript SK plasmid (Stratagene) as described (Ylä-
Hertuala *et al*: PNAS 1990; 87: 6959-6963). Briefly,
20   paraffin-embedded sections were pretreated with Proteinase K,
acetylated and hybridized using 35-S-UTP (DuPont, NEN)-
labeled riboprobes ($6x10^6$ cpm/ml) at 52°C for 16h. Final
wash after the hybridization was with 0.1xSSC at 60°C for 30
min. Autoradiography was used for the signal detection
25   (Eastman-Kodak NTB-2). Control hybridizations with a
nonhybridizing sense riboprobe (Ylä-Herttuala *et al*: PNAS
1990p 87: 6959-6963) gave negative results. Sections were
counter stained with hematoxylin. H-I were photographed
under polarized light epiluminescence. Magnification 200X
30   (A-G), 100x (H,I).

Example 1.3

**Administration of nitric oxide synth sis inhibitor L-NAME
abolish d th diff rence in intimal thickening b tween VEGF-
and lacZ-transf ct d art ries (Figur 3A).**

Gene transfer was carried out as described in Example 1.1.
L-NAME (70mg/kg/d) was given to the rabbits in drinking
water, starting one day before VEGF (n=5) or lacZ (n=5) gene
transfer. Animals were sacrificed 7 days after gene transfer
5   and analyzed for the intima/media thickness ratio and
histology as described above (Ylä-Herttuala *et al*
Arteriosclerosis 1986; 6: 230-236; Ylä-Herttuala *et al* PNAS
1990; 87: 6959-6963). Results are given as mean ± SD.

Example 1.4

10   **VEGF induces nitric oxide (NO) production in human umbilical
vein endothelial cells.**

Confluent cultures of HUVEC were washed twice with serum-free
medium and incubated with this medium either in the presence
of the concentrations of recombinant human VEGF indicated for
15   15 min, or with 10ng/ml VEGF for the times shown. In some
experiments, cells were pretreated for 1h with 100μM L-NAME
and subsequently treated either with or without 10ng/ml VEGF
for 10 min. The medium was removed (Figures 4A and B) and
cells were rapidly lysed at 4°C by addition of 10mM Tris/HCI
20   (pH 7.6), 5mM EDTA, 50mM NaCl, 30mM Sodium pyrophosphate,
50mM NaF, 0.1mM Na_3VO_4, 1mM PMSF and 1% Triton X-100 (lysis
buffer). Lysates were clarified by centrifugation at 15000
xg for 10min, and immunoprecipitations were performed by
incubating clarified lysates with PY20 anti-phosphotyrosine
25   mAb for 2h at 40°C. Immunoprecipitates were collected by
incubating lysates for a further 1 hour with protein A-
agarose. Immunoprecipitates were washed three times with
lysis buffer and proteins were then extracted with 2x SDS-
PAGE sample buffer. After SDS-PAGE immunoprecipitated
30   proteins were transferred to membranes and then immunoblotted
with PY20 mAb. Positions of molecular weight markers (kD)
are shown on the left, and positions of major tyrosine
phosphorylated bands are indicated in A by arrowheads.

See Figur s 4 C and D. Recombinant VEGF was added at the
concentration of 25 ng/ml for indicated times or at the
indicated concentrations for 10 minutes. Effect of L-NAME
(100 μM) pretreatment (1 hour) on the VEGF response was
5   measured after addition of 25 ng/ml VEGF for 10 minutes.
Nitrite production was measured using capillary detection
method (Leone et al, in methods in Nitric Oxide Research. Eds
Feelisch, M. and Stanler, J.S., John Wiley & Sons, New York
1996, pp 499-508). n=4-5 for each point, except for L-NAME
10   pretreatment, n=2. Results are given as mean ± SEM.

Example 2

**Use of plasmid/liposome complexes, Moloney murine leukemia
virus-derived (MMLV) retroviruses, pseudotyped vesicular
stomatitis virus protein-G (VSV-G)-containing retroviruses**
15   **and adenoviruses in delivering genes into the rabbit carotid
artery using a silastic collar applied to the adventitia.**

This method is used for gene transfer because 1) it provides
a gene delivery reservoir; 2) no intralumenal manipulations
are performed and endothelium remains anatomically intact
20   throughout; and 3) installation of the collar induces
arterial smooth muscle cell (SMC) proliferation and enhances
retroviral gene transfer efficiency where target cell
proliferation is required.

The transfer of the β-galactosidase (lacZ) marker gene to the
25   adventitia and outer media occurred with all gene transfer
systems. Adenoviruses also transferred the β-galactosidase
gene to some endothelial cells. After five days adenoviral
vectors produced the highest gene transfer efficiency with up
to 10% of cell showing β-galactosidase activity. Pseudotyped
30   VSV-G retroviruses were also effective in achieving gene
transfer in 0,1% of cells in the adventitia and outer media.
Plasmid/liposome complexes and MMLV retroviruses infected
<0.01% of cells. No adverse tissue reactions were observed

with any of the gene transfer systems.

Thus, replication-deficient adenoviruses, VSV-G pseudotyped
retroviruses and plasmid/liposome complexes can be used for
gene transfer to the arterial wall using the collar method.
5   Effects on medial SMC and even endothelium can be achieved
from the adventitial side.

Methods

Experimental animals. The Animal Care and Use Committee,
University of Kuopio, approved all animal procedures. 27 New
10 Zealand White rabbits (1.8-2.5kg) were used. The anesthetic
was fentanyl-fluanisone (0.3 ml/kg)/midatzolam (1 mg/kg) Ylä-
Herttuala *et al*: J. Clin Invest. 1995; 95: 2692-2698). A
midline neck incision exposed the left carotid artery. A
biologically inert 2cm silastic collar (MediGene Oy, Kuopio,
15 Finland) was positioned around the carotid artery so that it
touched the adventitia lightly at either end (Booth *et al*:
Atherosclerosis 1989; 76: 257-268). Gene transfer was
performed 4-5 days after the collaring operation. For gene
transfer animals were re-anesthetized. The collar, which had
20 been surgically re-exposed, was gently opened and filled with
500 μl of the gene transfer solution (see below). The
incision was closed and arteries later analyzed for gene
transfer efficiency.

Histological analysis: Collared arteries were carefully
25 removed and divided into three equal parts: the proximal
third was immersion-fixed in 4% paraformaldehyde/phosphate
buffered saline (pH 7.4) for 15 min, followed by embedding
into OCT compound (Miles Scientific, USA). The medial third
was immersion-fixed in 4% paraformaldehyde/phosphate buffered
30 saline (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) for
48 h and embedded in paraffin. The distal third was embedded
in OcT compound and processed for frozen sections. Ten
randomly selected sections were stained with X-gal for β-

galactosidase activity for 12 h and used for the
.determination of gene transfer efficiency (Nabel *et al*:
Science 1990; 249: 1285-1288; Ylä-Herttuala: J. Clin. Invest
1995; 95: 2692-2698). Gene transfer efficiency was

5   calculated as a percentage of the *β*-galactosidase-containing
cells as a proportion of the total number of nuclei in 20
randomly selected 100X fields. Randomly selected sections
from each third portion of the collared arteries were used
for immunocytochemistry and analysis of cell types and/or

10   intima/media thickness ratios (Booth *et al*, *supra*).

Cell types were identified using the following antibodies:
SMC: HHF-35 mAb (1:500 dilution, Enzo Diagnostics, USA), *α*-
actin mAb (1:1000 dilution; Sigma Chemical Co.); macrophages:
RAM-11 mAB (1:1000 dilution; Dako, USA), anti-CD68 mAb (1:250

15   dilution; Dako); endothelial cells: anti-CD31 mAb (1:50
dilution; Dako); polymorphonuclear leukocytes: anti-CD45 mAb
(1:100 dilution; Dako); and anti-rabbit T-cells: MCA 805 mAb
(1:1000 dilution; Dako). The avidin-biotin-horseradish
peroxidase system was used for signal detection (Vector

20   laboratories) (Ylä-Herttuala *et al*: J. Clin. Invest. 1995;
95: 2692-2698). After immunostaining, tissue sections were
counter stained with hematoxylin.

Determination of the proliferation index. The proliferation
index in the collared arteries was determined using the 5-

25   bromo-2'-deoxyuridine (BrdU) labelling (Soma *et al*:
Arterioscler. Thromb. 1993; 13: 571-578). Briefly, New
Zealand white rabbits (n=12) were injected with BrdU (40
mg/kg body weight) 3 h before sacrifice. Carotid arteries
were fixed in 70% ethanol overnight and embedded in paraffin.

30   Serial sections (20 sections per animal) were stained to
detect BrdU using FITC-labeled anti-mouse IgG (Dako),
following propidium iodide staining of the nuclei. The
labelling index was calculated as the percentage of the BrdU-
positive nuclei. Contralateral carotid arteries were sham-

35   operated and used as controls.

Gen transfer vectors.

Plasmid/liposomes: pCMV-β-galactosidase (lacZ) expression
plasmid (Promega) was complexed with Lipofectin reagent (BRL)
as follows: 25 μg plasmid was slowly mixed with 25 μl
5   Lipofectin reagent while diluted to 500 μl with Ringer
solution. No precipitates were observed in the
plasmid/Lipofectin solution. The mixture was left to stand
at room temperature for at least 15 min and used for gene
transfer within two hours. Plasmid preparations were checked
10   for the absence of lipopolysaccharide contamination (Limulus
assay, Sigma Chemical Co.).

Retroviruses: LacZ-containing pLZRNL MMLV retroviruses (Ylä-
Herttuala et al: J. Clin. Invest. 1995; 95: 2692-2698;
Miyanohara et al: PNAS 1988; 85: 6538-6542) or LacZ VSV-G
15   pseudotyped retroviruses (Yee et al: PNAS 1994; 91: 9564-
9568) were used for the studies. In both, the expression of
lacZ is driven by the 5' LTR. Replication-deficient LZRNL
amphotrophic retroviruses were packaged in PA317 cells and
used at a titer of 5x10^5 cfu/ml as described (Ylä-Herttuala
20   et al: J. Clin. Invest. 1995; 95: 2692-2698). Replication-
deficient VSV-G pseudotyped retroviruses were produced in 293
GP cells using transient transfection (Yee et al, Supra.
Pseudotyped retroviruses were concentrated using
ultracentrifugation and used at a titer of 1x10^7 cfu/ml.
25   Before use, retroviral preparations were checked for the
absence of any bacteriological contaminants or helper viruses
(Yee et al, supra).

Adenoviral vectors: Replication-deficient E1-deleted
adenoviruses were used for the studies (Gosh-Choudhury et al:
30   Gene 1986; 50: 161-171; Simari et al: J. Clin. Invest. 1996;
98: 225-235). Nuclear targeted β-galactosidase cDNA under a
β-actin-promoter and a CMV enhancer was cloned into the E1-
deleted region of the adenoviral genome using homologous
recombination (Gosh-Choudhury et al: Gene 1986; 50: 161-171;

Simari *et al*: J. Clin. Invest. 1996; 98: 225-235).
Replication-deficient adenoviruses were produced in 293 cells
and concentrated by ultracentrifugation. Titers of $1x10^9$
pfu/ml were used for the gene transfer experiments.

5   Adenoviral preparations were analyzed for the absence of
helper viruses or bacteriological contaminants (Gosh-
Choudhury *et al*, *supra*).

Results

The adventitial collar led to neointimal hyperplasia 7-14
10 days after the operation (Fig. 5A). The endothelium remained
anatomically intact throughout the studies (Fig. 6A). BrdU
labelling indicated a peak proliferation index of 23% 3 days
after the operation (Fig. 5B). The neointima was exclusively
composed of SMC (Fig. 6B). Plasmid/liposome complexes led to
15 a detectable gene transfer into the adventitia and outer
media (Fig. 6D), with an efficiency of less than 0.01%.
Untransfected or liposome-treated collared arteries showed no
staining for β-galactosidase activity (Fig. 6I).

Adventitial retroviral gene transfer was not successful
20 without the collar probably because retroviral gene transfer
only occurs in proliferating cells. The gene transfer
efficiency with replication-deficient MMLV retroviruses was
low (less than 0,01%) (Fig. 6E). Gene transfer efficiency
with VSV-G pseudotyped retroviruses was 0.1% (Fig. 6F). With
25 MMLV and VSV-G retroviruses β-galactosidase staining was
observed in the adventitia and outer media.

Replication-deficient adenoviruses gave efficient gene
transfer (Fig. 6G, H), with β-galactosidase staining detected
in the adventitia and outer media. Interestingly, staining
30 was also observed in some endothelial cells and in some
intimal cells. Since the lacZ adenovirus construct contained
a nuclear localization signal for β-galactosidae, intense X-

gal staining was located in the nuclei of the transfected
cells (Fig. 6H). Gene transfer efficiency was approximately
10%, as estimated from the total number of stained nuclei in
the analyzed sections. Some inflammatory cells were seen in
VSV-G retrovirus and adenovirus-transfected arteries Figs 6I
and J. No inflammatory cells were seen in the
plasmid/liposome transfected arteries.

FIGURE LEGENDS

Figure 1

VEGF gene transfer reduces intimal thickening in rabbit
carotid arteries 7 days after the gene transfer. (A)
intima/media area ratios in VEGF (black bars) and lacZ (open
bars) transfected arteries. (B) VEGF transgene mRNA
expression in VEGF and lacZ transfected arteries as analyzed
using RT-PCR. Lane 1: Control PCR without cDNA template;
Lane 2: VEGF transfected artery with an expected 547nt
amplified fragment indicating the expression of the transgene
; Lane3: lacZ transfected artery showing no transgene
expression; Lane 4: Same as lane 2 but 5' primer omitted;
Lane 5: Same as lane 2 but 3' primer omitted; Lane 6:
positive control plasmid for the transgene. L: DNA size
markers (1kb ladder, BRL).

Figure 2

Representative micrographs of the characteristics of rabbit
carotid arteries 7 days after VEGF or lacZ gene transfer
using the collar model. (A-G) immunostainings of the
transfected arteries: (A) Control artery transfected with
lacZ-plasmid/liposomes (SMC-specific MAb HHF-35, 1:500
dilution, Enzo Diagnostics) showing a typical intimal
thickening; (B) Artery transfected with VEGF
plasmid/liposomes (SMC-specific MAb HHF-35) where only
limited intimal thickening is seen; (C) Endothelium was

present in all studied vascular segments (s rial section to
A, EC-specific MAb CD 31, dilution 1:50, DAKO); (D)
Endothelial staining (serial section to B, MAb CD31); (E) No
evidence of inflammation was detected in VEGF-and lacZ-
5   transfected arteries (Macrophage-specific MAb RAM-11 dilution
1:500, DAKO); (F) Neovascularization in the adventitia of
VEGF-transfected artery 14 days after gene transfer
(Hematoxylin-eosin staining); (G) Non-immune control for the
immunostainings (first antibody omitted). (H-I) *In situ*
10   hybridization with anti-sense VEGF riboprobes. (H) Low level
VEGF mRNA expression in adventitia of control lacZ-
transfected arteries; (F) VEGF mRNA expression in adventitia
of VEGF-transfected arteries.

Figure 3

15   Administration of nitric oxide synthesis inhibitor L-NAME
abolished the difference in intimal thickening between VEGF·
transfected arteries (black bar) and lacZ-transfected (open
bar) arteries.

Figure 4

20 . VEGF induces tyrosine phosphorylation and nitric oxide
production in HUVEC. (A) VEGF induces phosphorylation of a
major 205kD protein corresponding to VEGF receptor
(arrowheads indicate major tyrosine phosphorylated proteins);
(B) Time course of tyrosine phosphorylation. NO synthesis
25   inhibitor L-NAME abolishes the response to VEGF; (C) Time
course and (D) dose-response of nitrite production after
addition of VEGF to HUVEC.

Figure 5

Neointimal thickening and proliferation indices in the rabbit
30   carotid arteries after positioning of a silastic collar
around the carotid artery. A:Intima/media thickness ratio

after the operation. Arteries with the collar (□); sham-
operated control arteries (■); B: BrdU labeling indexes in
intima (□) and media (■) of the collared arteries. Values
were obtained from 20 serial cross sections per rabbit (mean
5   ± SD, three animals per time point).

Figure 6

β-galactosidase gene transfer into the collared rabbit
carotid arteries using plasmid/liposome complexes,
replication-deficient MMLV retroviruses, VSV-G pseudotyped
10   retroviruses and adenoviruses. Gene transfer was done on day
five after the collar operation. Arteries were analyzed five
days after the gene transfer for general histology, cell
types and β-galactosidase activity using immunocytochemistry
and X-gal staining. (A-C) Immunocytochemical stainings of
15   serial sections of a Plasmid/liposome-transfected artery: A:
Endothelium remained anatomically intact throughout the
studies (endothelial staining with mAb CD31, dilution 1:50);
B: The majority of cells in intima and media were smooth
muscle cells (smooth muscle staining with mAb HHF-35,
20   dilution 1:500); C: Non-immune control for the
immunostainings. (D-H) Arteries transfected with various
gene transfer constructs: D: Plasmid/liposome complexes (25
μg lacZ plasmid, 25 μg Lipofectin reagent in 500 μl Ringer
solution); E: MMLV retroviruses (500 μl pLZRNL retrovirus,
25   titer 5 x 10^5 cfu/ml); F: VSV-4 pseudotyped retroviruses
(500μl pL ZRNL+G retrovirus, titer 1 x 10^7 cfu/ml) G:E1-
deleted adenoviruses (500 μl nuclear targeted pCMVBA-lacZ Ad5
adenovirus, titer 1 x 10^9 pfu/ml); H: Higher magnification of
G showing intense staining of the nuclei in the adventitia
30   with the nuclear-targeted lacZ construct and X-gal staining
of some endothelial cells. Original magnification 40x (A-
C,G); 100x (D-F) ·

Figure 7

Schematic view of a preferred implant (collar) of the
invention in place around a blood vessel (coronal section).

Figure 8

5   Schematic view of a preferred implant (collar) of the
invention in place around a blood vessel (longitudinal
section).

SEQUENCE INFORMATION

These sequences include a 26 amino acid presequence (see
10   above). References to these sequences throughout this
specification are references both to the forms shown here
including the presequence and to the forms without
presequences.

SEQ ID No. 1 and 2
15   cDNA (SEQ ID No. 1) and encoded amino acid (SEQ ID No. 2)
ꞌ   sequence of VEGF-121

```
ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTC GCC TTG CTG CTC
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

     TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
20   Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

     GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
     Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

25   CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
     Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

     TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
     Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu
```

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

5   ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

10
GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA TGT GAC AAG
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Cys Asp Lys

CCG AGG CGG
15 Pro Arg Arg

SEQ ID No. 3 and 4
cDNA (SEQ ID No. 3) and encoded amino acid (SEQ ID No. 4)
sequence of VEGF-165
20 Lysine at position 141 may be replaced by asparagine (see
above).

ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTC GCC TTG CTG CTC
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

25 TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
30 Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
5   Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA CCC TGT GGG
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Pro Cys Gly

10 CCT TGC TCA GAG CGG AGA AAG CAT TTG TTT GTA CAA GAT CCG CAG ACG
Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp Pro Gln Thr

TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC AAG GCG AGG CAG
Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln
15

CTT GAG TTA AAC GAA CGT ACT TGC AGA TGT GAC AAG CCG AGG CGG
Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg

SEQ ID No. 5 and 6
20 cDNA (SEQ ID No. 5) and encoded amino acid (SEQ ID No. 6)
sequence of VEGF-189

ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTC GCC TTG CTG CTC
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
25 Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

30 CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro Leu

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
5 Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
10 Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA AAA TCA GTT
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val

CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT
15 Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr

AAG TCC TGG AGC GTG CCC TGT GGG CCT TGC TCA GAG CGG AGA AAG CAT
Lys Ser Trp Ser Val Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His

TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA
20 Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr

GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC
Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys

25 AGA TGT GAC AAG CCG AGG CGG
Arg Cys Asp Lys Pro Arg Arg

SEQ ID No. 7 and 8
cDNA (SEQ ID No.7) and encoded amino acid sequence (SEQ ID No.
30 8) of VEGF-206

ATG AAC TTT CTG CTG TCT TGG GTG CAT TGG AGC CTC GCC TTG CTG CTC
Met Asn Phe Leu Leu Ser Trp Val His Trp Ser Leu Ala Leu Leu Leu

TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ATG GCA GAA GGA
Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala Glu Gly

GGA GGG CAG AAT CAT CAC GAA GTG GTG AAG TTC ATG GAT GTC TAT CAG
Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp Val Tyr Gln

5

CGC AGC TAC TGC CAT CCA ATC GAG ACC CTG GTG GAC ATC TTC CAG GAG
Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp Ile Phe Gln Glu

TAC CCT GAT GAG ATC GAG TAC ATC TTC AAG CCA TCC TGT GTG CCG CTG
Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser Cys Val Pro L u

10

ATG CGA TGC GGG GGC TGC TGC AAT GAC GAG GGC CTG GAG TGT GTG CCC
Met Arg Cys Gly Gly Cys Cys Asn Asp Glu Gly Leu Glu Cys Val Pro

ACT GAG GAG TCC AAC ATC ACC ATG CAG ATT ATG CGG ATC AAA CCT CAC
15 Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His

CAA GGC CAG CAC ATA GGA GAG ATG AGC TTC CTA CAG CAC AAC AAA TGT
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys

20 GAA TGC AGA CCA AAG AAA GAT AGA GCA AGA CAA GAA AAA AAA TCA GTT
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val

CGA GGA AAG GGA AAG GGG CAA AAA CGA AAG CGC AAG AAA TCC CGG TAT
Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr

25

AAG TCC TGG AGC GTG TAC GTT GGT GCC CGC TGC TGT CTA ATG CCC TGG
Lys Ser Trp Ser Val Tyr Val Gly Ala Arg Cys Cys Leu Met Pro Trp

AGC CTC CCT GGC CCC CAT CCC TGT GGG CCT TGC TCA GAG CGG AGA AAG
30 Ser Leu Pro Gly Pro His Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys

CAT TTG TTT GTA CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC
His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn

ACA GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT

Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr

TGC AGA TGT GAC AAG CCG AGG CGG
Cys Arg Cys Asp Lys Pro Arg Arg

CLAIMS

1.   Use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of blood vessel, of vascular endothelial growth factor (VEGF) protein or a nucleic acid encoding VEGF protein.

2.   Use according to claim 1 for the treatment or prevention of intimal hyperplasia induced by a surgical procedure.

3.   Use according to claim 1 or 2 wherein the blood vessel is an artery.

4.   Use according to claim 2 or 3 wherein the surgical procedure is angioplasty, coronary bypass surgery, surgical anastomosis or endarteriectomy.

5.   Use according to claim 3 wherein the surgical procedure is a carotid artery endarteriectomy or a balloon angioplasty.

6.   Use according to claim 1 in the treatment or prevention of hyperplasia which is associated with pulmonary artery hypertension.

7.   Use according to any one of the preceding claims wherein the treatment comprises administering VEGF protein.

8.   Use according to claim 7 wherein the VEGF protein is human VEGF protein.

9.   Use according to claim 9 wherein the human VEGF protein has the sequence of SEQ ID No. 2, 4, 6 or 8.

10.   Use according to any one of the preceding claims wherein the treatment comprises administering a nucleic acid encoding VEGF at or near the site of the hyperplasia to be treated or

prevented, which nucleic acid is expressed to generate VEGF.

11.   Use according to claim 10 wherein the nucleic acid is a
DNA having the sequence of SEQ ID No. 1, 3, 5 or 7.

12.   Use according to claim 10 or 11 wherein the nucleic acid
5   is administered by means of a vector.

13.   Use according to claim 12 wherein the vector is a viral
vector.

14.   Use according to claim 13 wherein the viral vector is an
adenovirus, a retrovirus, a pseudotyped retrovirus, a
10   herpesvirus, a vaccinia virus or a baculovirus.

15.   Use according to claim 12 wherein the vector is a non-
viral vector.

16.   Use according to claim 15 wherein the non-viral vector
is an oligonucleotide, a plasmid, a liposome, a liposome-
15   protein complex, an immunoliposome, a liposome-protein-
polylysine derivative, a water-oil emulsion, a polyethylene
imine, a dendrimer or a plasmid/liposome complex.

17. Use according to claim 16 wherein the liposome is a
cationic liposome or a pH-sensitive liposome.

20   18.   Use according to any one of the preceding claims wherein
the VEGF or nucleic acid encoding VEGF is introduced into an
implant.

19.   Use according to claim 18 wherein the implant is placed
at or near the site of the hyperplasia to be treated or
25   prevented and releases the VEGF or nucleic acid encoding
VEGF.

20.   Use according to claim 18 or 19 wherein the implant is a

silastic implant or a biodegradable implant.

21.   Use according to any one of claims 18 to 20 wherein the implant is in the form of a collar for fitting around the artery at or near the site of the hyperplasia to be treated or prevented.

22.   An implant comprising VEGF protein or a nucleic acid encoding VEGF protein.

23.   An implant according to claim 22 comprising a nucleic acid encoding VEGF.

24.   An implant according to claim 23 wherein the nucleic acid is associated with a vector.

25.   An implant according to claim 24 wherein the vector is as defined in any one of claims 13 to 17.

26.   An implant according to any one of claims 18 to 25 wherein the VEGF nucleic acid has the sequence of SEQ ID No. 1, 3, 5 or 7.

27.   An implant according to claim 18 which the implant comprises VEGF protein.

28.   An implant according to claim 27 wherein the VEGF protein is human VEGF protein.

29.   An implant according to claim 28 wherein the human VEGF protein has the sequence of SEQ ID No. 2, 4, 6 or 8.

30.   An implant according to any one of claims 22 to 29 which allows the VEGF protein or nucleic acid into direct contact with the blood vessel.

31.   An implant according to any one of claim 22 to 30

comprising an outer wall substantially impermeable to the
VEGF nucleic acid or protein.

32. An implant according to claim 30 or 31 wherein, in use,
the extremities of the implant are in contact with the wall
5   of the blood vessel.

33. An implant according to any one of claims 22 to 32 which
is in the form of a collar for fitting around a blood vessel
at or near the site of hyperplasia to be treated or
prevented.

10 34. An implant according to any one of claims 22 to 33 which
is silastic or biodegradable.

35. A method of treating or preventing intimal hyperplasia
of a blood vessel comprising administering to a patient in
need of such treatment an effective non-toxic amount of VEGF
15 or of a nucleic acid encoding VEGF.

36. A method according to claim 32 which comprises
implanting an implant as defined in any one of claims 22 to
34 into a patient in the vicinity of the hyperplasia to be
treated, thereby to effect delivery of VEGF or a nucleic acid
20 encoding VEGF.

37. A kit for the treatment on prevention of intimal
hyperplasia which comprises: (i) VEGF protein or nucleic acid
as defined in any one of claims 1 to 17; and (ii) an implant
into which the VEGF protein or nucleic acid may be
25 introduced.

38. A kit according to claim 37 wherein the implant is as
defined in any one of claims 30 to 34.

39. A kit according to claim 37 or 38 wherein the VEGF
protein or nucleic acid is provided in association with a

pharmaceutically acceptable carrier.

40.   Use, in the manufacture of a medicament for the treatment or prevention of intimal hyperplasia of a blood vessel of Nitric Oxide synthase (NOS) or a nucleic acid
5   encoding NOS wherein the NOS protein or nucleic acid is provided in an implant as defined in any one of claims 30 to 32.

41.   Use according to claim 40 wherein the Nitric Oxide synthase is inducible nitric oxide synthase (iNOS) or the
10   nucleic acid encodes iNOS.

42.   A method of treating or preventing intimal hyperplasia of a blood vessel comprising implanting an implant as defined in claim 40 or 41 into a patient in the vicinity of the hyperplasia to be treated or prevented, thereby to effect
15   delivery of NOS or nucleic acid encoding NOS said implant comprising an effective non-toxic amount of NOS, optionally iNOS.

43.   Use according to claim 40 or 41 or a method according to claim 42 wherein the nucleic acid is associated with a vector
20   as defined in any one of claims 13 to 17.

44.   A kit for the treatment or prevention of intimal hyperplasia which comprises: (i) NOS, optionally iNOS, protein or nucleic acid as defined in any one of claims 40, 41 or 43; and (ii) an implant into which the NOS protein or
25   nucleic acid may be introduced.

45.   A kit according to claim 44 wherein the implant is as defined in any one of claims 30 to 34.

46.   An implant as defined in any one of claims 30 to 34 comprising NOS protein or nucleic acid as defined in any one
30   of claims 40, 41 or 43.
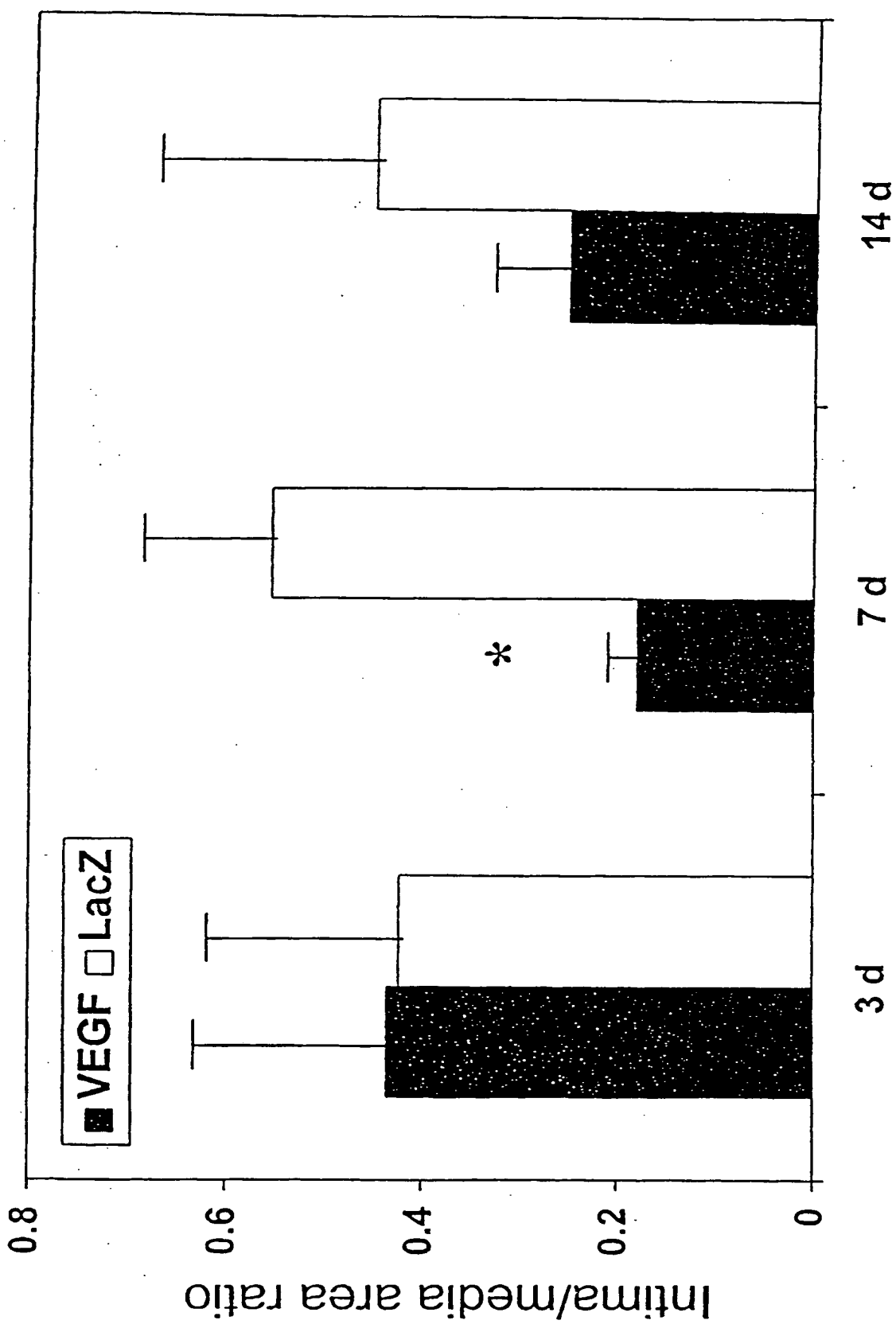
FIGURE 1A
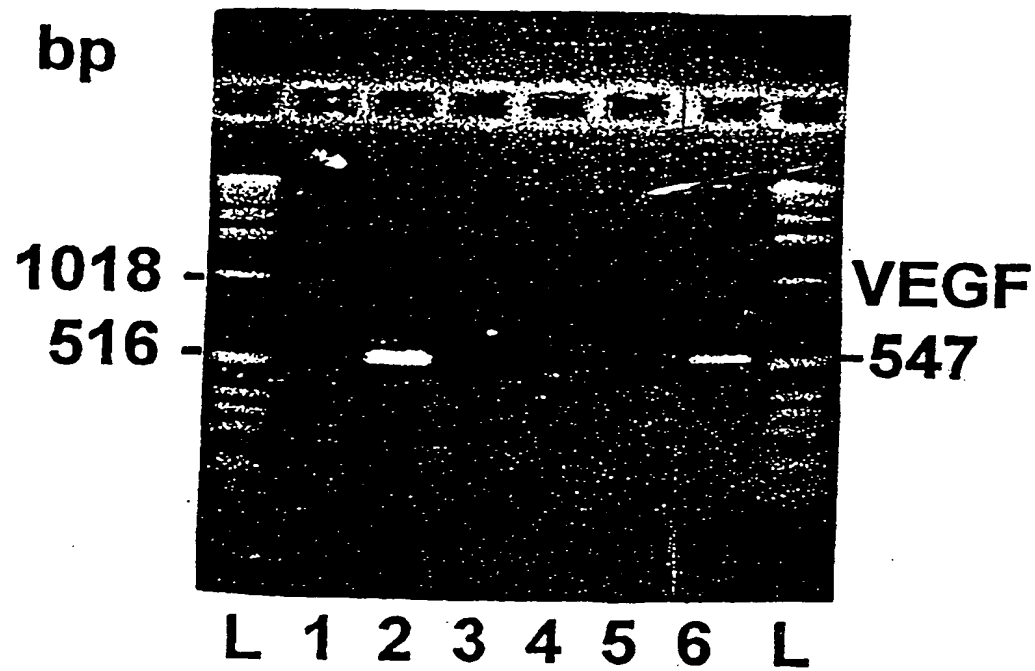
FIGURE 1B

FIGURE 2A
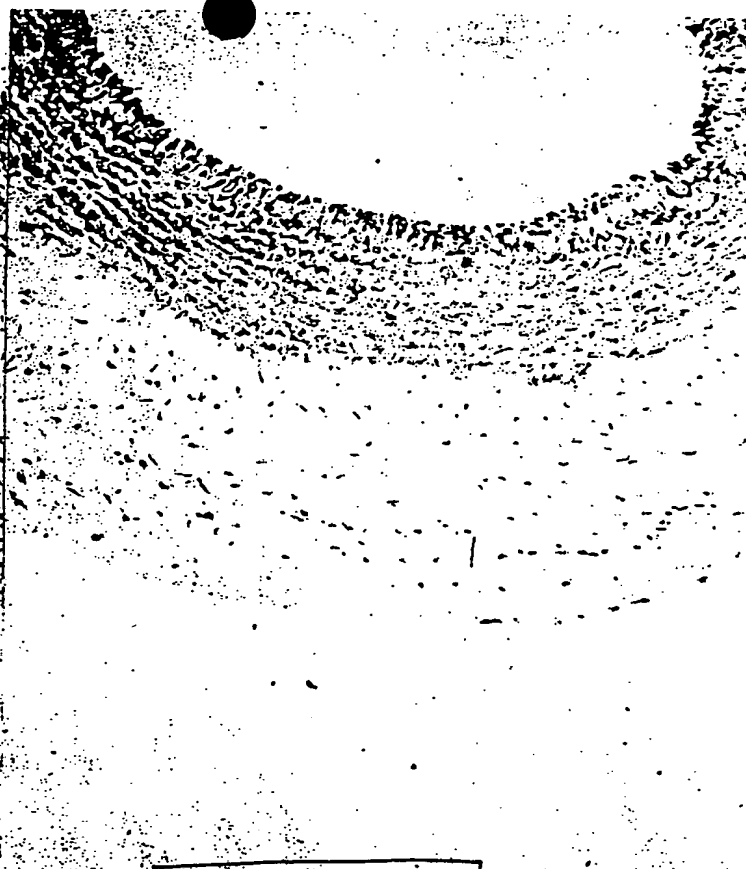
FIGURE 2B
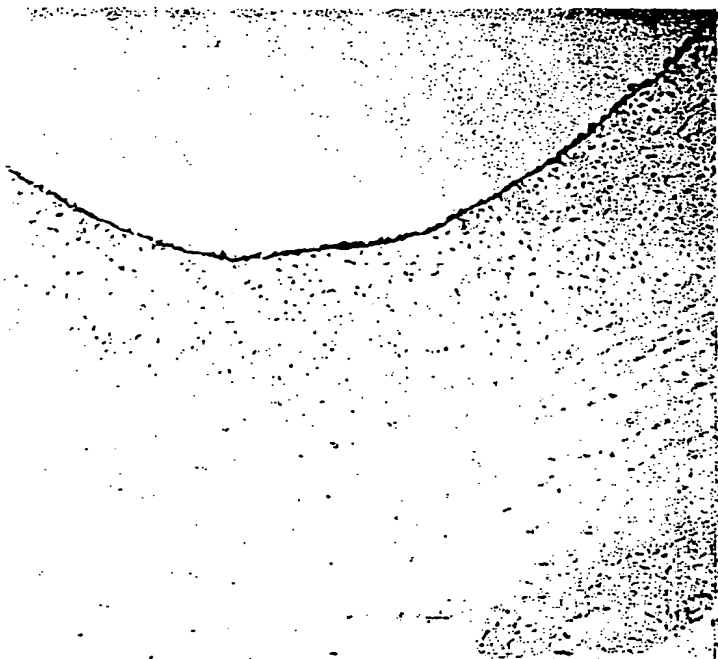
FIGURE 2C

FIGURE 2D
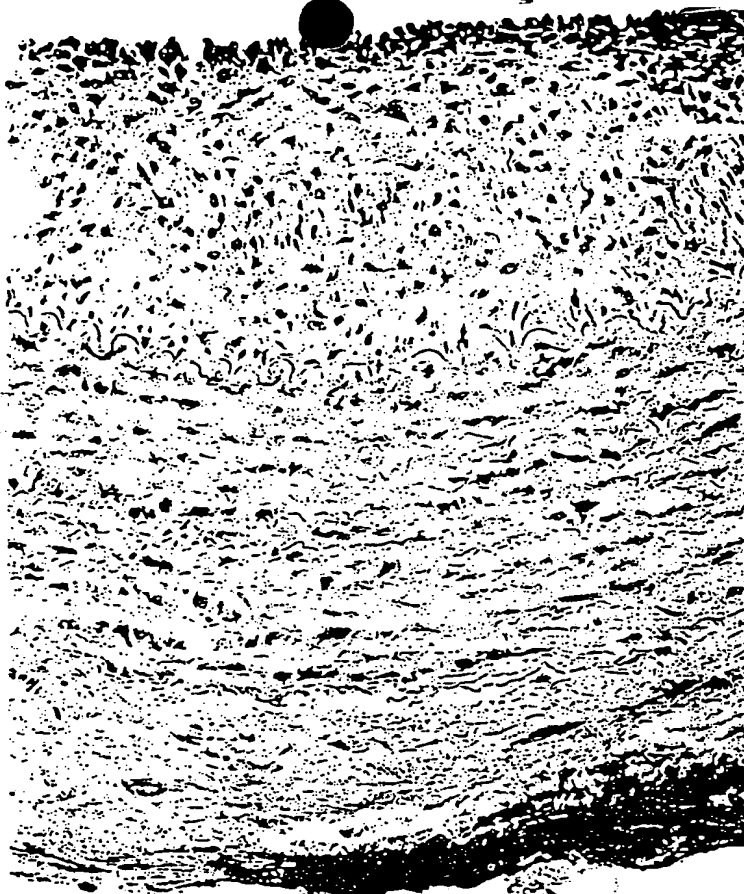
FIGURE 2 E
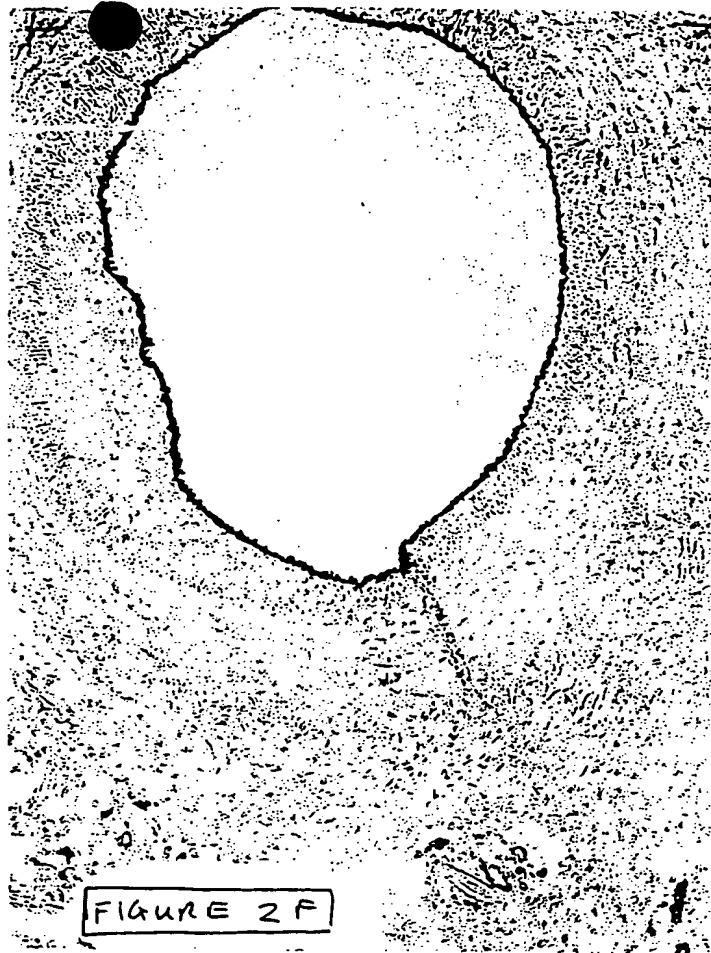
FIGURE 2 F

FIGURE 2 G

FIGURE 3

A

205
190
145
125
100

200 —

97 —

66 —

0   0.5   1   2.5   10   25

VEGF, ng/ml

B

200 —

97 —

66 —

0   0.5   1   5   10   0   10

Time, min   L-NAME

C

Nitrite (μM)

2,1
2
1,9
1,8
1,7
1,6
1,5

0   50   100   150

Time (min)

D

Nitrite (μM)

2,2
2
1,8
1,6

0   2,5   25   25
                    + L-NAME

VEGF (ng/ml)
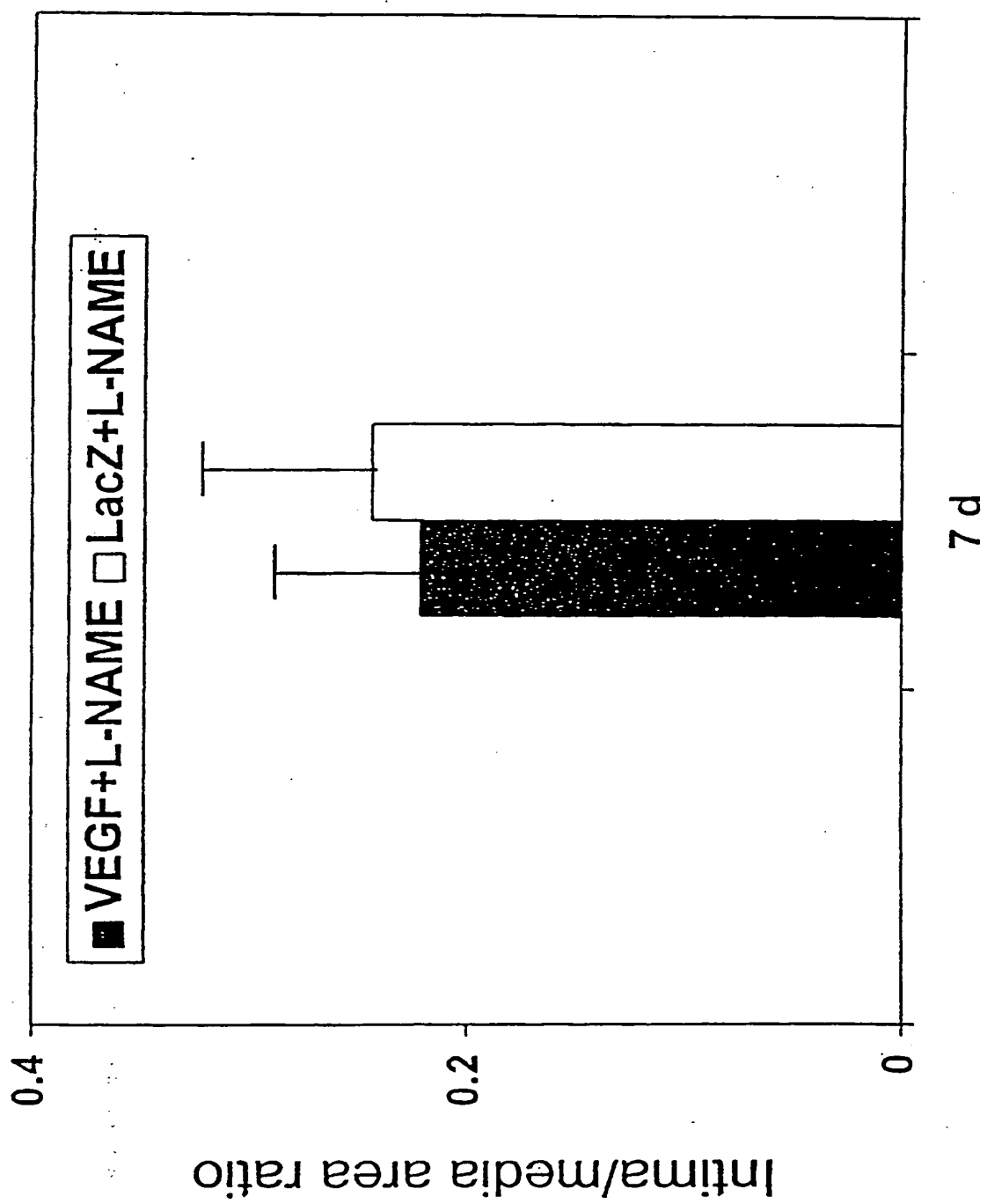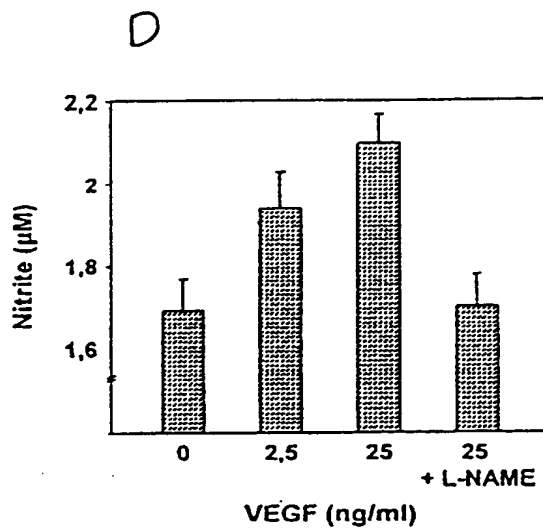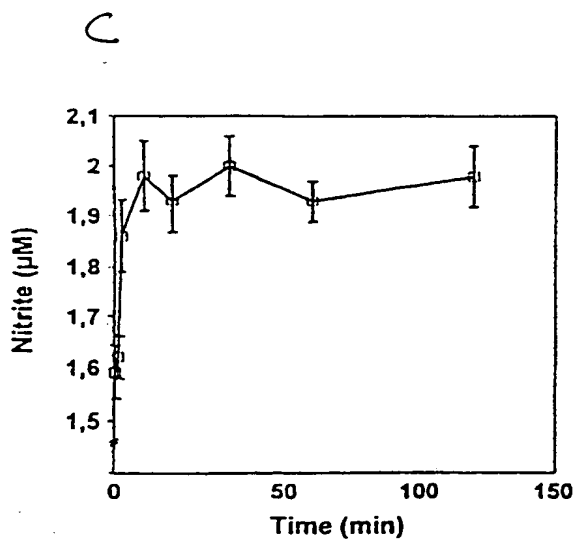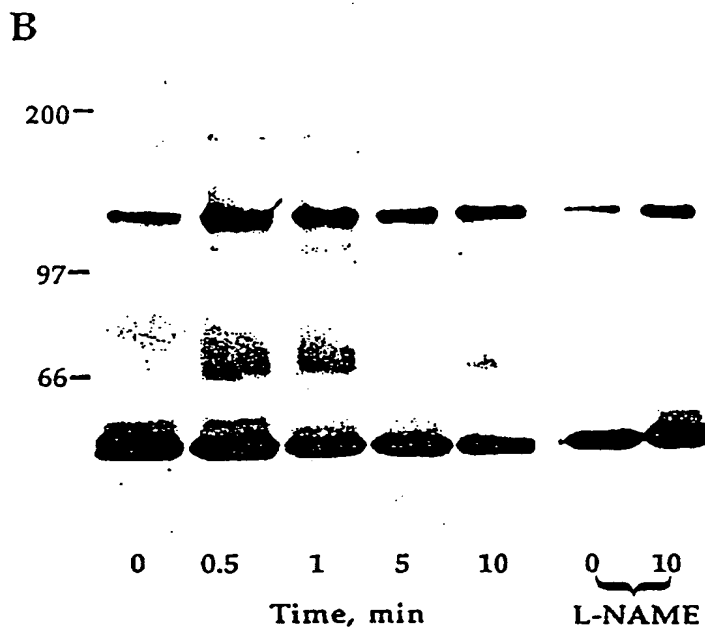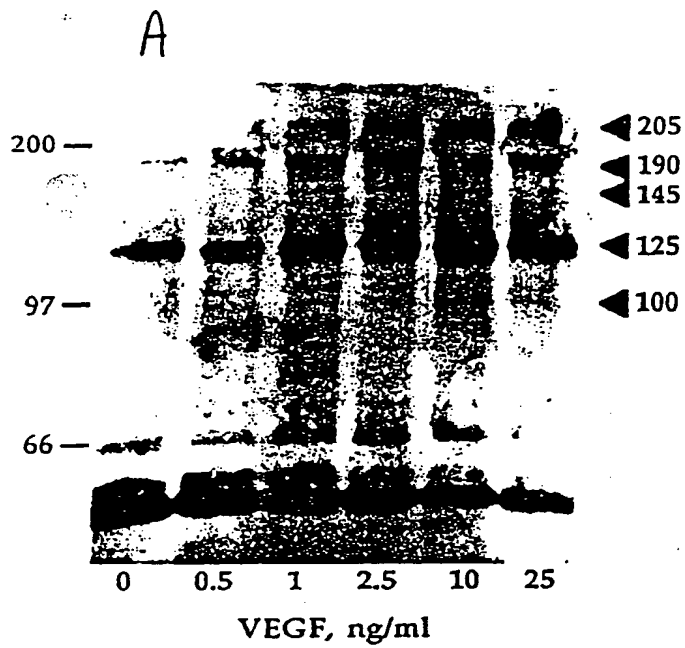
FIGURE 4

FIGURE 5A

FIGURE 5B
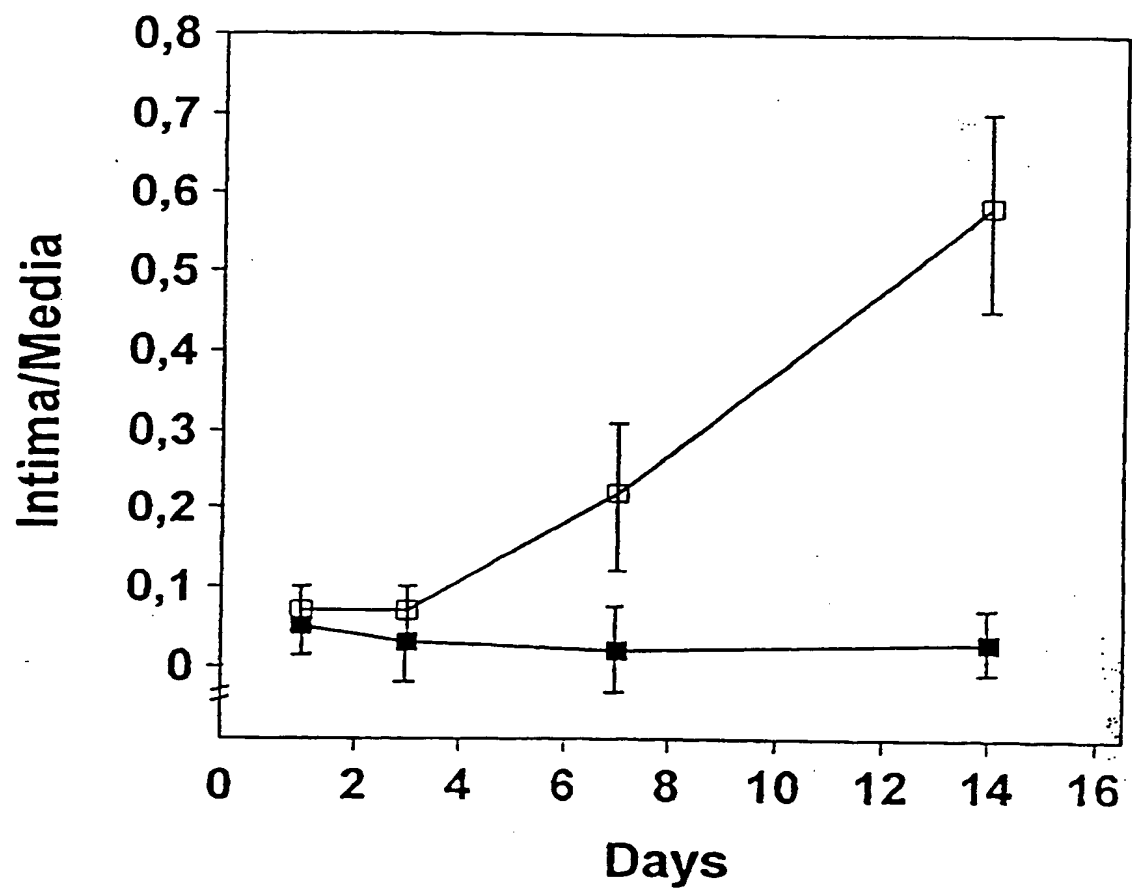
FIGURE 6A
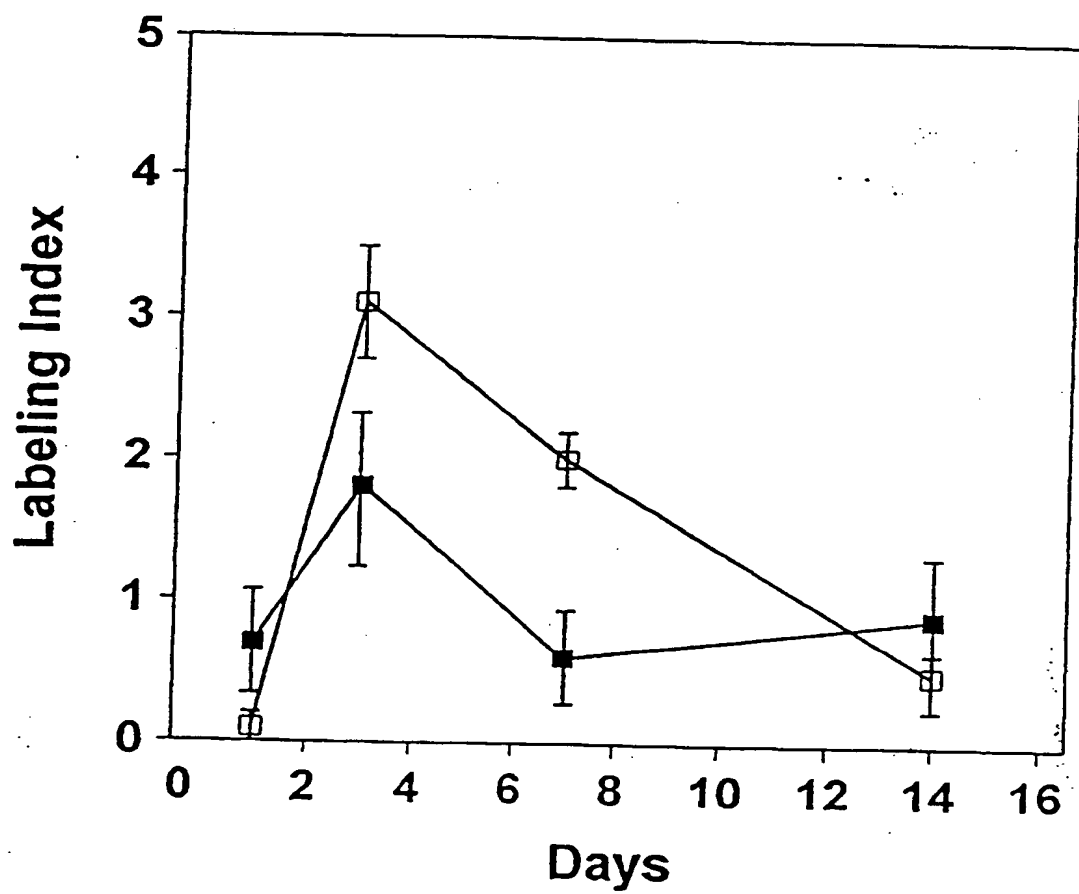
FIGURE 6B
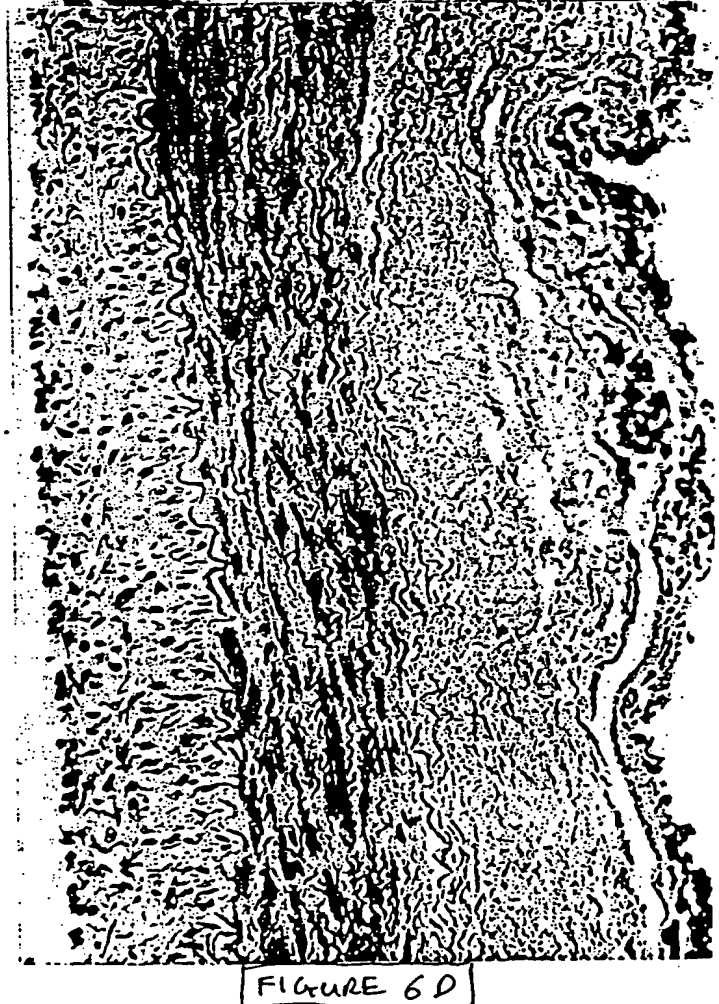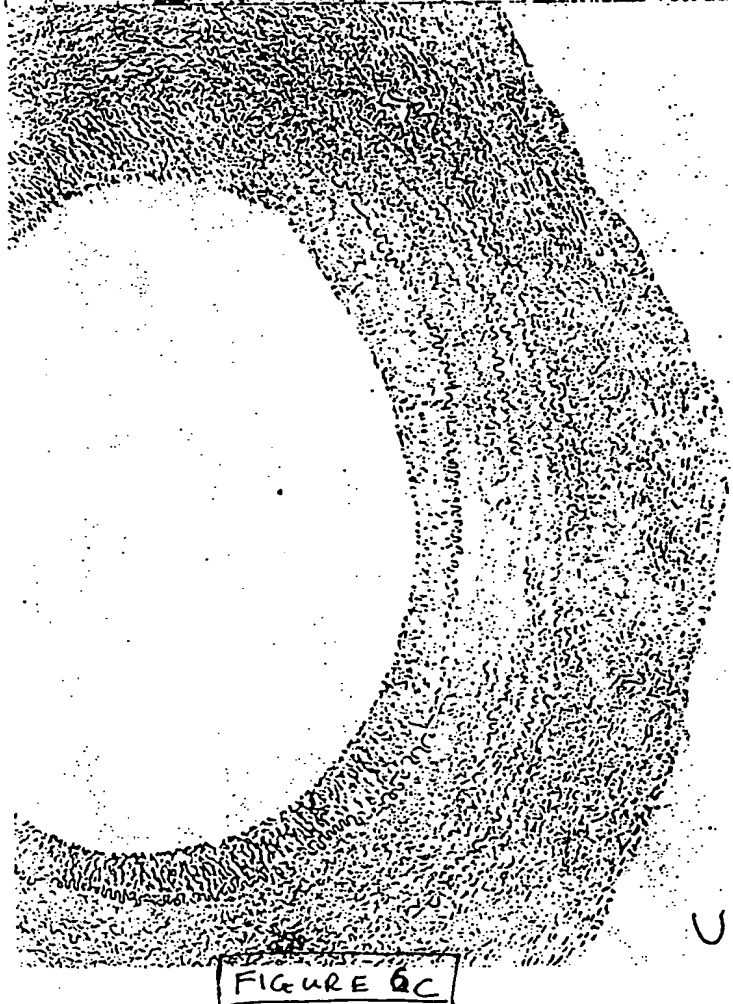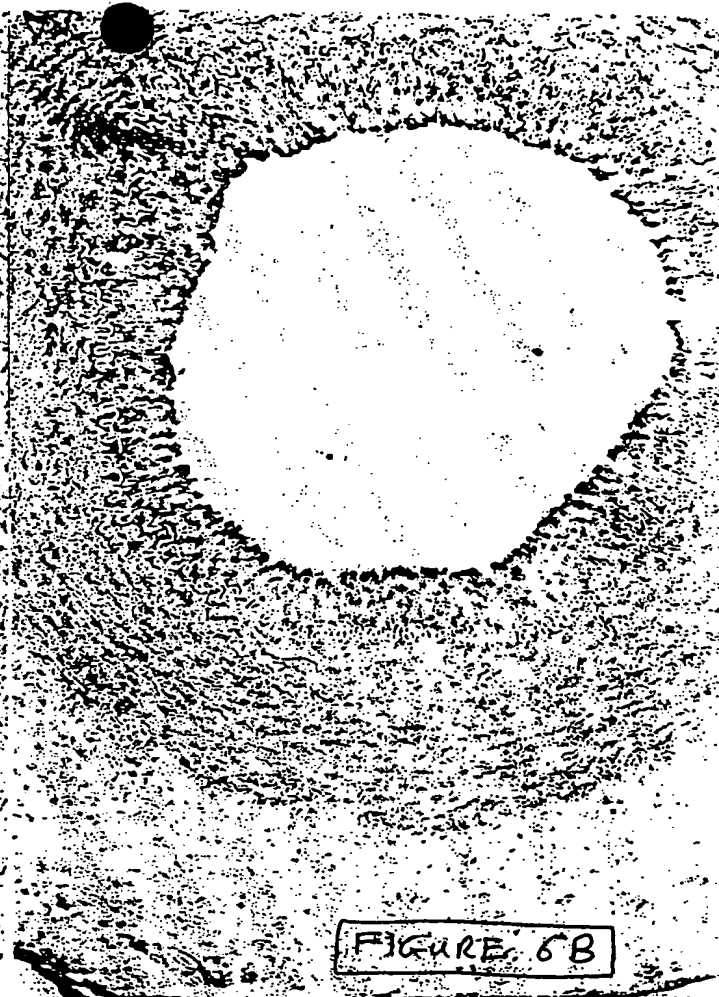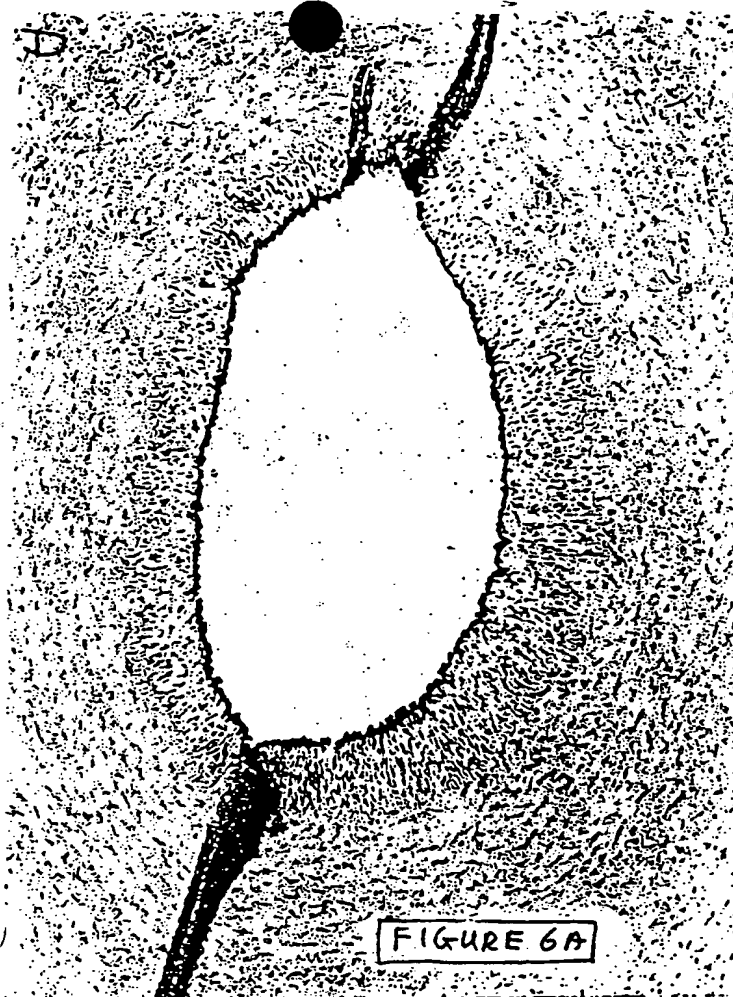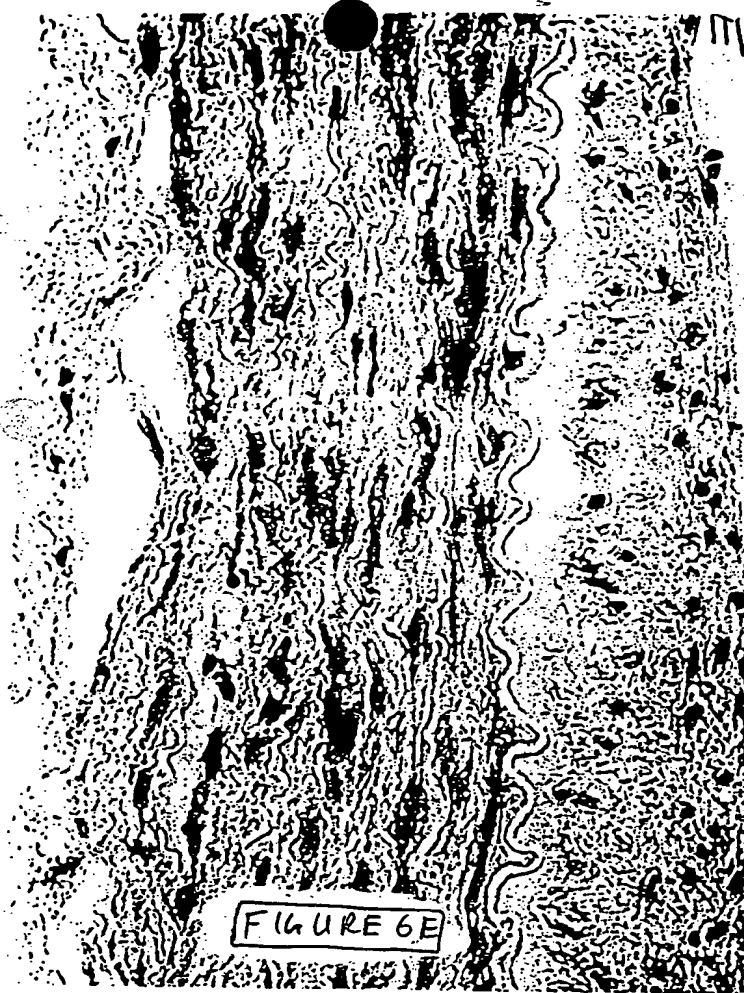
FIGURE 6C
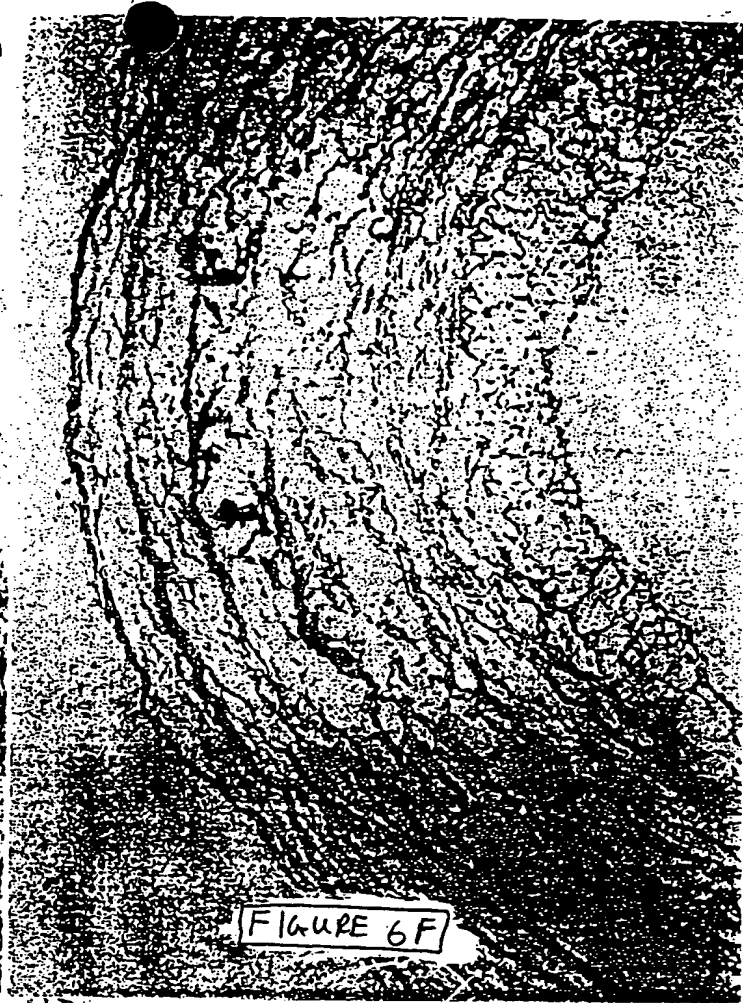
FIGURE 6D

FIGURE 6E

FIGURE 6F

FIGURE 6G

FIGURE 6H

Wall of collar

Blood

Vessel wall

Void space

Material for
entry into the arterial
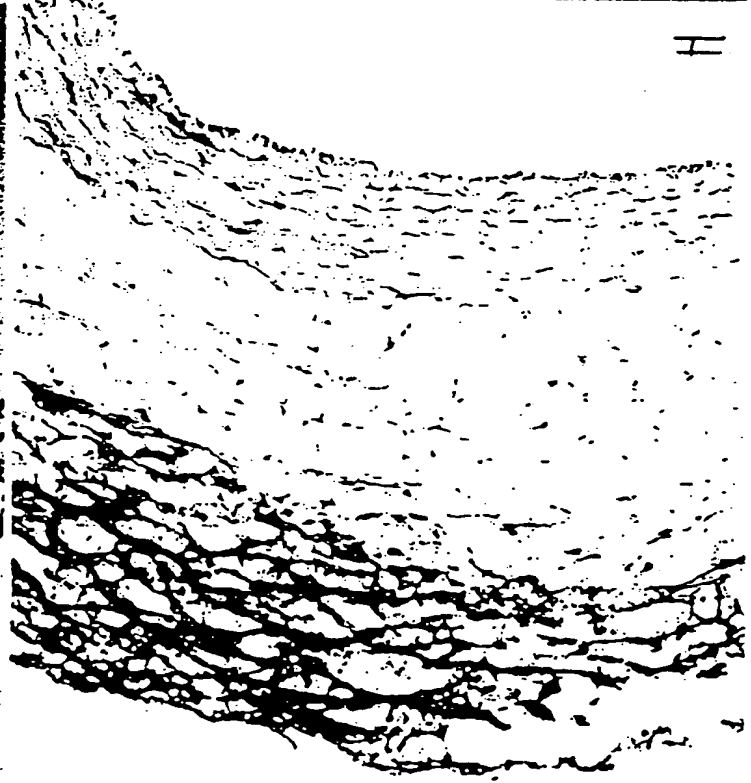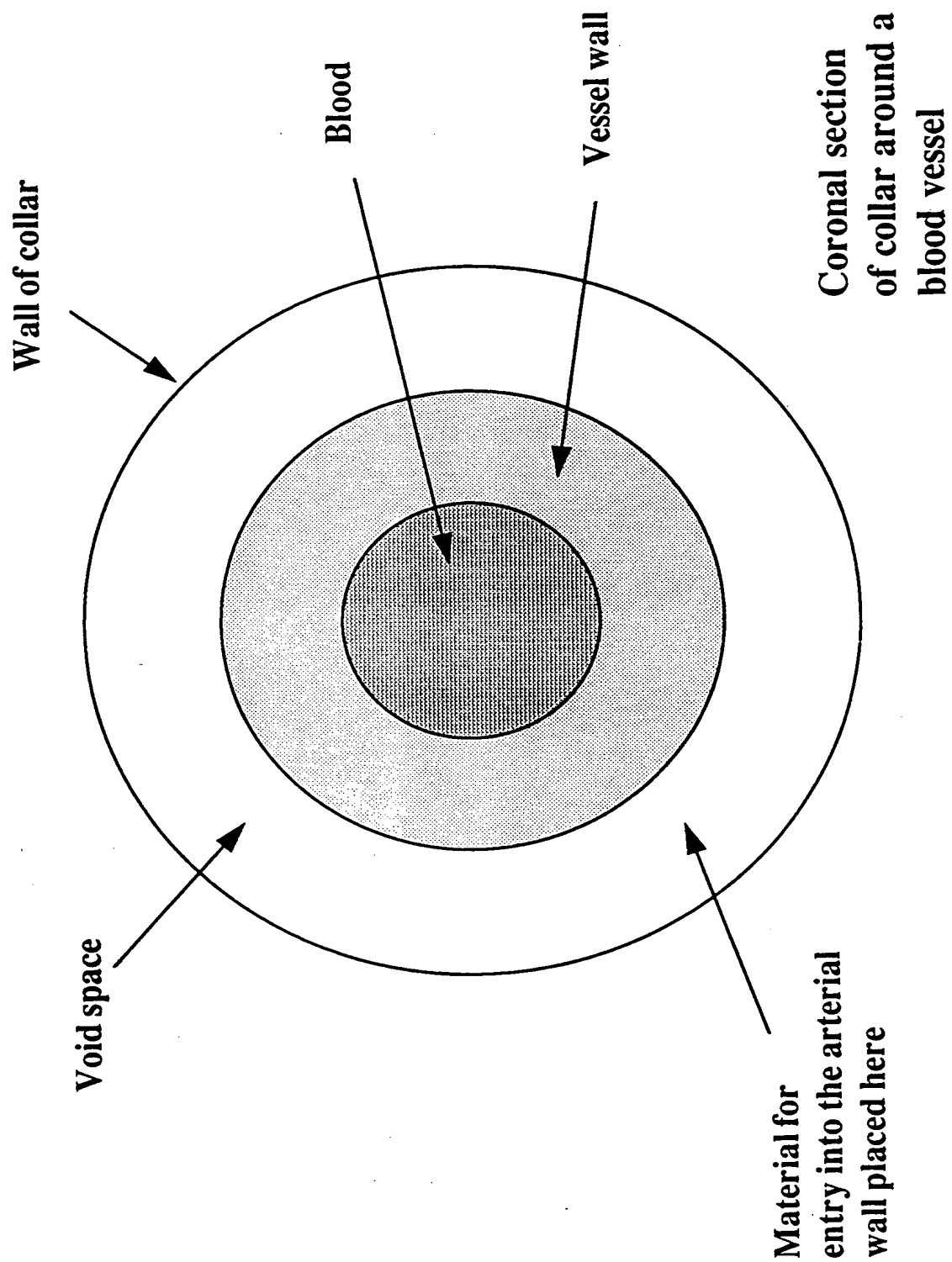wall placed here

Coronal section
of collar around a
blood vessel
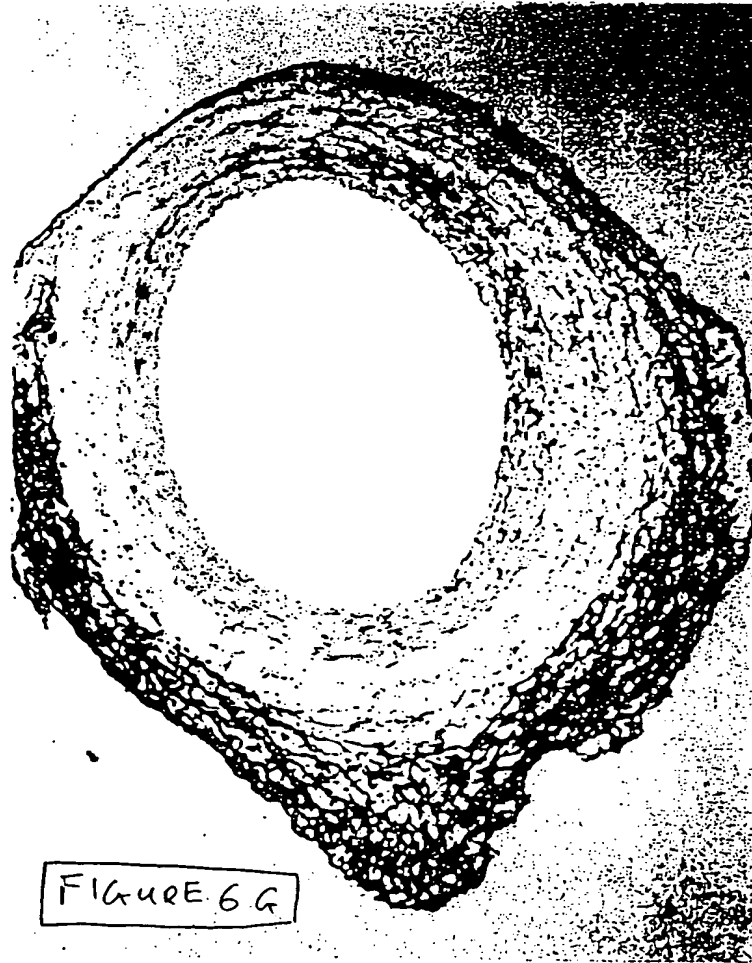
FIGURE 7

Longitudinal section of an example of a collar-reservoir

Material, which may be biodegradable, forming a hollow container around the blood vessel

Material for entry into vessel wall contained here

Vessel wall

Blood

Void

The collar touches the vessel wall at its extremeties

FIGURE 8